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PRINCIPAL INVESTIGATOR: Khandan Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: University of Albany, SUNY
Albany, New York 12201-0509

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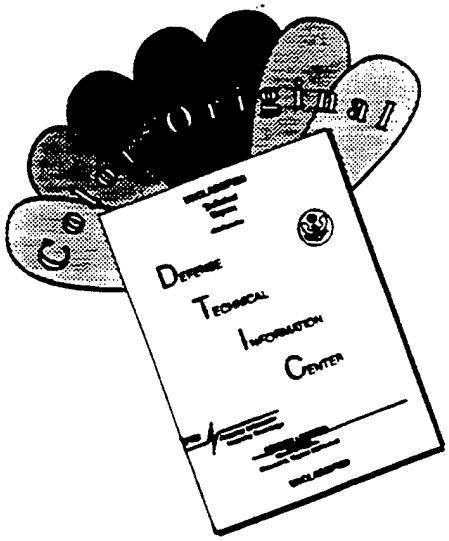

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Albany, New York 12201-0509

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Cyclin E is an important regulator of cell cycle progression that together with cyclin-dependent kinase 2 (cdk2) is crucial for the G1/S transition during the mammalian cell cycle. Previously, we showed that severe overexpression of cyclin E protein in tumor cells and tissues results in the appearance of lower molecular weight isoforms of cyclin E which together with cdk2 can form a kinase complex active throughout the cell cycle. In this study we report that one of the substrates of this constitutively active cyclin E/cdk2 complex is pRb in populations of breast cancer cells and tissues which also overexpress p16. In these tumor cells and tissues, we show the expression of p16 and pRb are not mutually exclusive. Overexpression of p16 in these cells results in sequestering of cdk4 and cdk6, rendering cyclin D1/cdk complexes inactive. However pRb appears to be phosphorylated through out the cell cycle following an initial lag revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes which functionally inactivate pRb. In addition the constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor but not the normal cell cycle. These observations suggest that overexpression of cyclin E in tumor cells which also overexpress p16, can bypass the cyclin D/cdk4-cdk6/p16/pRB feedback loop, providing yet another mechanism by which tumors can gain a growth advantage.

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5. Introduction:

The overall purpose of this 4 year study is to use the altered expression of cyclin E as a diagnostic/prognostic marker and to investigate the mechanisms and repercussions of this alteration in breast cancer.

Cyclins are prime cell cycle regulators and central to the control of cell proliferation in eukaryotic cells via their association with and activation of cyclin-dependent protein kinases 1-7 (cdks) (reviewed in (1-6). Cyclins were first identified in marine invertebrates as a result of their dramatic cell cycle expression patterns during meiotic and early mitotic divisions (7-10). Several classes of cyclins have been described and are currently designated as cyclins A-H, some with multiple members. Cyclins can be distinguished on the basis of conserved sequence motifs, patterns of appearance and apparent functional roles during specific phases and regulatory points of the cell cycle in a variety of species. The cdk partners of several of these cyclins have also been identified: Cyclin A forms a complex with cdc2 (cdk1) and cdk2, and is required both at mitosis and DNA replication (11-14); cyclin B forms a complex solely with cdc2 and is required for entry into mitosis, (reviewed in (3); cyclin D1, a cyclin active in the G1 phase of the cell cycle, forms complexes primarily with cdk4 and cdk6, while cyclin E, another G1 type cyclin, forms a complex with only cdk2 (3, 4, 15-19). Lastly, cyclin H has been shown to form a complex with cdk7 and, together, they comprise the cdk-activating kinase (CAK) protein complex which activates the nascent cyclin/cdk complex via phosphorylation (20, 21). Cyclin binding to a cdk enables the kinase to become active, initiating a complex kinase cascade that directs the cell into DNA synthesis and/or mitosis, reviewed in (15, 22).

An additional layer of cell cycle regulation has emerged with the discoveries of low molecular weight cdk inhibitors (CKIs) which represent a novel mode of negative regulation (23-25). The first class of these inhibitors, p21, was simultaneously characterized in several laboratories as the major p53 inducible gene (WAF1) (26-28), as a CDK inhibitor protein (CIP1, p21, and $p20^{CAP1}$) (29-31), as a protein highly expressed in senescent fibroblasts (sdi) (32), and

as a melanoma differentiation associated gene (mda6) (33). In normal fibroblasts, this protein has been shown to be associated with and inhibit various cyclin-cdk complexes, including cdk2 associated with cyclins A and E, cdk4 associated with D-type cyclins (29, 30, 34-36), and is also found weakly associated cdc2-cyclin B (34). This protein which represents one of the major p53 inducible genes, is also induced during differentiation. It most likely acts as a general purpose brake used during terminal differentiation and p53 directed DNA damage control (37). The second protein in this family, $p27^{KIP1}$, is both structurally and functionally similar to p21. $p27^{KIP1}$ was identified simultaneously as a protein associated with inactive cyclin E-cdk2 complexes in TGFß treated and contact inhibited cells (38, 39) and as a protein that interacts with cyclin D1-cdk4 complexes (40). TGFß arrests certain cell types in G1 and p27 is thought to be a cellular mediator for this anti-proliferative signal (41). Hence, p21 and p27 may function similarly to inhibit cdk activity and proliferation in response to different environmental stimuli.

A second, structurally and functionally distinct family of CKIs is comprised of p16, p15 and their homologous (34, 42-45). Structural features of these Ink4 (for inhibitor of cdk4) proteins include 4 ankyrin like repeats which are postulated to be involved in mediating protein-protein interactions (43, 46). Curiously these CKIs share significant homology to the Notch proteins involved in the differentiation and fate determination of cells during embryogenesis (42). Inhibitors of this family bind cdk monomers (cdk4 or cdk6) rather than cyclin-cdk complexes (34, 46). It is believed that binding of ink4 proteins to cdks prevents and/or disrupts cyclin-cdk complex formation thereby negating cdk activity. p16 and p15 proteins, encoded on human chromosome 9, have been the subjects of intense study as this genomic region is frequently mutated in a variety of tumor cell lines and fewer tumor tissue specimens (34, 46). As their alternate names imply (MTS1 and MTS2 for multiple tumor suppressor) p16 and p15 are postulated to function as growth inhibitory tumor suppressor molecules.

The connection between cyclins CKIs and cancer has been substantiated with the D type cyclins (6, 9, 22, 47). Cyclin D1 was identified simultaneously by several laboratories using independent systems: It was identified in mouse macrophages due to its induction by colony

stimulating factor 1 during G1 (48). It was also identified in complementation studies using yeast strains deficient in G1 cyclins (15, 49); as the product of the bcl-1 oncogene (50), and as the PRAD1 proto-oncogene in some parathyroid tumors where its locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene (48, 51-53). In centrocytic B cell lymphomas cyclin D1 (PRAD1)/BCL1 is targeted by chromosomal translocations at the BCL1 breakpoint, t(11;14)(q13;q32) (54, 55). Furthermore, the cyclin D1 locus undergoes gene amplification in mouse skin carcinogenesis, as well as in breast, esophageal, colorectal and squamous cell carcinomas (56-62). Several groups have examined the ability of cyclin D1 to transform cells directly in culture with mixed results (9, 51, 61, 63-67). However, the overexpression of cyclin D1 was recently observed in mammary cells of transgenic mice and results in abnormal proliferation of these cells and the development of mammary adenocarcinomas (68). This observation strengthens the hypothesis that the inappropriate expression of a G1 type cyclin may lead to loss of growth control.

Cyclins D2 and A have also been implicated in oncogenesis. The cyclin D2 gene appears to be the integration site of a murine leukemia provirus in mouse T cell leukemias, resulting in its overexpression (69). Cyclin A was found to be the site of integration of a fragment of the hepatitis B virus genome in a hepatocellular carcinoma (70). Cyclin A is also associated with the adenovirus transforming protein E1A in adenovirus transformed cells (71, 72).

The linkage between oncogenesis and the cell cycle was recently reinforced by correlating the deranged expression of cyclins to the loss of growth control in breast cancer (58, 73). Using proliferating normal versus human tumor breast cell lines in culture as a model system, several changes were seen in all or most of these lines. These include increased cyclin mRNA stability, resulting in overexpression of mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, leading to the deranged order of appearance of mitotic cyclins prior to G1 cyclins in synchronized tumor cells. The most striking abnormality in cyclin expression was that of cyclin E. Cyclin E protein not only was overexpressed in 10/10 breast tumor cell lines but it was also present in lower molecular weight isoforms than that found in normal cells (73). The relevance of cyclin

derangement to *in vivo* conditions, was directly examined by measuring the expression of cyclin E protein in tumor samples versus normal adjacent tissue obtained from patients with various malignancies (74). These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition, the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA or c-erb B2. These observations strongly suggested the use of cyclin E as a new prognostic marker. These findings were corroborated by immunocytochemical detection of cyclin E which detects tumor proliferation and deregulated cyclin expression. The mechanism of the cyclin E alteration is in part a result of its deregulation in breast cancer. The alteration of cyclin E in breast cancer have been recently further characterized and reveal that while cyclin E is cell cycle regulated in normal cells it is present constituitively and in an active cdk2 complex in synchronized populations of breast cancer cells. Two novel truncated variant forms of cyclin E mRNA as detected by RT-PCR were also identified which are ubiquitously detected in normal and tumor cells and tissues. These variant forms of cyclin E can give rise to an active cyclin/cdk2 complex *in vitro*, but they do not seem to be translated in normal cells.

During the first year of this application we have used cyclin E antibody as a prognostic marker for breast cancer by analyzing 400 more breast tumor tissue specimens for the alterations in cyclin E protein. We also compared the expression of cyclin E to expression of other tumor markers such as cyclin D1 and erbB2. We also have obtained information on the steroid receptor status as well as DNA ploidy and proliferation rate of each tumor tissue sample. During the second year of the grant we have analyzed all the Western blot analysis with cyclin E and other markers, obtained outcome information on all the patients including TNM staging, final diagnosis, treatments given, as well as disease state at last contact. Having all this information we are now equipped to perform statistical analysis on these samples and evaluate the role of cyclin E as a prognosticator for breast cancer.

During the first year of the grant we initialized some studies on the characterization of the mechanism of alteration of cyclin E using cultured cells. During the second year of the grant we documented that cyclin E is in fact deregulated in breast cancer and such deregulation gives rise to redundancy in function. We show that under conditions where cyclin E is overexpressed it can act redundantly and replace cyclin D as well as cyclin A in breast cancer cell lines. We also document that such redundancy is also seen in tumor tissue specimen. During the second year of the application we also begun characterizing the regulation of CKIS including $p21^{CIP1}$ in normal versus tumor cells.

6: Body (Results)

During the second year of this grant application we continued studies as outline in the first 2 Specific Aims of our application and initiated studies on Specific Aims 3 and 4. The first study which consisted of using cyclin E antibody as a diagnostic/prognostic marker for breast cancer and as described in the first aim of our application will take 3 years to complete. During the first two years we collected 550 tumor tissue samples from breast cancer patients diagnosed with different stages of breast cancer ranging from pre-malignant to highly invasive. We extracted RNA, DNA and protein from most of these samples. Due to limited sample size received for each patient (i.e 0.1-0.2 g of tissue), protein was initially extracted from all samples and if there was tumor sample left over, DNA and RNA were also extracted. The protein extracts from all 500 samples were then subjected to Western blot analysis and the expression of cyclin E was compared and correlated with other known prognostic markers examined in the same samples. The prognostic markers include, cyclin D1, erbB-2, as well as PCNA to determine the proliferative activity of these samples. We also obtained information on the estrogen and progesterone receptor status of each sample as well as ploidy and proliferation rate as measured by Ki-67. In the second year of this study we analyzed the results obtained in the first year by quantitating the levels of cyclin E in each tumor specimen with that of cyclin D1, erbB2 and PCNA. These analysis were done by performing densitometric scanning on each lane of each gel with each antibody for each patient

sample using at least two autoradiographs with different exposures. Such laborious analysis were necessary to accurately determine the level of cyclin E protein in every patient and correlate the alteration of cyclin E protein from each patient to the stage of their disease. We also have been in contact with the 20 hospitals where these samples were obtained, and have been successful in collecting the following information on each patient: final diagnosis, TNM staging, treatment given, and final outcome (i.e quality of survival). We are now in the process of inputting all this information in our data base and will perform statistical analysis on whether cyclin E alteration is correlated with the stage of breast cancer and whether such a correlation is predictive of outcome. We anticipate to complete this portion of the study during year 3 (i.e current year) of the application, and if we require additional patient samples, we can analyzed them in year 4 of the application.

The second study involved the utilization of cyclin E deletional mutations to detect early metastatic breast cancer. In the first year of this study we determined that the cyclin E forms we had referred to as deletional mutations turned out not to be deletional mutations, but rather splicing variants of cyclin E that are present in both normal and tumor cell lines and tissue samples. However, these truncated forms of cyclin E will help us decipher the mechanisms of alteration of cyclin E in breast cancer. During the first year we investigated the cell cycle expression of cyclin E in normal versus tumor cells at the level of protein kinase activities and documented that cyclin E is constituitively present and active during the tumor but not normal cell cycles. We also showed that in vitro the truncated forms of cyclin E are capable of binding to cdk2 and activating the kinase complex and phosphorylating substrates such as Histone H1. These results were summarized in an article which appeared in Oncogene in 1995 and a reprint is enclosed in the appendix. During the second year of this application we investigated the consequences of cyclin E overexpression and showed that due to its constitutive overexpression in tumor cells it act redundantly and replace both cyclin D and A for function. These studies fall under task 3 and 4 of our application. To this end we have also investigated the role of p21 and the other cyclin-dependent kinase inhibitors in breast cancer as outlined in the last two tasks of our application.

Overexpression of p16 and absence of cyclin D1/Cdk4 -cdk6 complexes, in a breast cancer cell line with functional retinoblastoma protein: A panel of 13 breast cell lines were surveyed for the correlation of p16 and Rb status as well as association of p16 and cyclin D1 with cdks 4 and 6 (Figure 1) The cell lines used include three proliferating normal mammary epithelial cell strains obtained from reduction mammoplasties and used at early passages, one near diploid normal-immortalized breast epithelial cell line and 9 tumor cell lines with different estrogen receptor, and p53 status, and cyclin E levels as outlined in table 1.

We examined the expression of pRb by direct immunoblotting with a monoclonal antibody where the presence of functional pRb is inferred from the presence of higher molecular weight hyperphosphorylated forms of the protein. These analysis revealed that besides three tumor cell lines (figure 1A, lanes 8, 11 and 12-i.e MDA-MB-436, HBL-100, and Hs-578T), where pRb is either mutated (75), inactive due to its binding to SV40 large T-antigen, or not expressed, pRb is present and functional in all the other cell lines examined. Furthermore, in all the pRB positive cell lines, there are at least two pRb bands present representing different phosphorylation states of pRB. (Due to different levels of pRb expression in each of the cell lines longer exposures were used to evaluate presence of slower migrating/functional form of pRb specifically in lanes 1, 2 and 5- data not shown). Next, we correlated the expression of p16 levels with pRb status and found that p16 is overexpressed in three cell lines (figure 1A, lanes 6, 8 and 11), two of which Rb has been functionally compromised (i.e. MDA-MB-436 and HBL-100). Curiously, in MDA-MB-157 which contains a wild-type pRB, p16 is also markedly overexpressed (figure 1A, lane 6). Hence MDA-MB-157, in which cyclin E is severely overexpressed [Table 1, (43)], is one exception to the reciprocal p16/Rb correlation rule.

Since, overexpression of cdk4, cdk6 or cyclin D1 could counteract the inhibitory effect caused by the over-abundance of p16, we also measured the relative levels of these proteins in all 13 cell lines (Fig. 1A). Western blot analysis with cyclin D1, cdk4 and cdk6 revealed that these proteins were not overexpressed in MDA-MB-157 cell line relative to the other 12 cell lines examined, suggesting that the overexpressed p16 may adequately sequester cdk4 and cdk6 away

from cyclin D1, rendering it inactive. To test this hypothesis we performed a series of 2 step immunoprecipitations followed by Western blot analysis (Fig. 1B). When p16 immunoprecipitates were separated on denaturing gels, transferred to PVDF membrane, and blotted with antiserum to cdk4 or cdk6, p16 was capable of forming a complex with both cdk4 and cdk6 in the three tumor cell lines where p16 is overexpressed. Curiously, p16 was also capable of forming a complex with cdk6 in normal breast cell strains where no overexpression of p16 or cdk6 were noted. However, cyclin D1 immunoprecipitates which were separated and blotted with antibodies to cdk4 or cdk6 revealed that in the normal cell strains cyclin D1 formed a complex with cdk4 and cdk6 suggesting that p16 did not completely sequester these kinases from cyclin D1. On the other hand, in tumor cells were p16 is overexpressed, no complexes were formed between cyclin D1 and cdk4 or cdk6, suggesting that in these three tumor cell lines enough p16 is overexpressed to sufficiently sequester cdk4 and cdk6 away from cyclin D1 preventing it from forming complexes with these kinases (Fig. 1B). Collectively these data provide evidence for the absence of cyclin D1/CDK complexes in a breast cancer cell line with a functional retinoblastoma protein.

Cyclin E associated kinase phosphorylates pRb in the absence of cyclin D1/cdk4 or cyclin D1/cdk6 complexes in tumor cells: To examine the cell cycle regulation of pRb in normal and tumor cells we synchronized both cell lines by double thymidine block and analyzed the pattern of pRb expression and phosphorylation by Western blot analysis (Fig 2A). Synchrony of both cell types at several times after release from the block was monitored by flow cytometry[1] (Fig 2C). At various times after release from treatment for synchronization, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to pRb, cyclins E and A (Fig 2A). In normal 76N cells, the pattern of synthesis and phosphorylation of pRb as well as

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[1]Although the doubling times of the normal 76N and tumor MDA-MB-157 cells are slightly different (27 and 36 h, respectively), their flow cytometry profiles are similar, indicating equal DNA content distribution in different cell cycle phases (data not shown).

expression of cyclin E and cyclin A proteins is consistent with that seen for other normal cell types with levels rising prior to S phase and oscillating thereafter in the cell cycle (19, 76, 77). In addition pRb is present mainly in the hyperphosphorylated form at G1/S boundary up to G2, where the levels drop, to resume again at G1. Furthermore, there is only one major form (i.e., 50KDa) of cyclin E protein detected and there is a shift in the timing of when cyclin E versus cyclin A appears in the cell cycle of these normal epithelial cells. However, in the tumor cells, pRb and cyclin E proteins do not appear to be cell cycle regulated. pRb is induced and phosphorylated shortly after release from thymidine block and remains in that phosphorylated state through out the cell cycle. In addition, multiple isoforms of cyclin E protein are present with similar signal intensities and banding patterns during the time intervals examined. In the same tumor cell extracts, cyclin A protein is cell cycle regulated with peak levels coinciding with peak S and early M phase. Hence, it appears that in this tumor cell line, pRb and cyclin E are abnormally regulated during the cell cycle.

To decipher whether cyclin E-associated kinase is responsible for the phosphorylation of pRb, cells were immunoprecipitated with cyclin E antibody and used in kinase assays with either histone H1 or a recombinant GST-Rb fusion protein as substrates. In normal cells, cyclin E associated kinase is capable of phosphorylating histone H1 and is cell cycle regulated, coinciding with the levels of cyclin E protein expression (Fig 2A). However the same cyclin E immunoprecipitates prepared from normal cells were not capable of phosphorylating GST-Rb. In tumor cells, on the other hand, cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle resulting in a constitutive pattern of histone H1 and GST-Rb phosphorylation. Lastly, the timing of pRB expression in the tumor cell cycle (Fig. 2A) is similar to the timing of phosphorylation of GST-Rb by cyclin E immunoprecipitates. These observations suggest that cyclin E protein, which is constituitively expressed in the cell cycle of tumor cells, results in an active kinase complex throughout the cell cycle capable of not only phosphorylating histone H1, but also GST-Rb. Hence in tumor cells which overexpress p16,

resulting in the inactivation of cyclin D1/CDK4 or cyclin D1/cdk6 complexes, pRb can still get phosphorylated by cyclin E/associated kinase.

Overexpression of cyclin E and p16 in breast tumor tissues is correlated with functional pRb: Since the lack of inverse association of pRb and p16 was observed in only one of 3 breast tumor cell lines overexpressing p16 (Figure 1A), we were interested in deciphering the frequency at which such a phenomena would occur in breast tissue samples. As such we examined 20 tumor tissue specimen obtained from breast cancer patients. Table 2 lists estrogen and progesterone status, ploidy, and proliferation index expression as measured by immunoflouresence with the respective antibodies followed by image analysis as previously described (78, 79). We also analyzed the expression of cyclin E, p16 and pRb in these samples by Western blot analysis. The results revealed that cyclin E was severely overexpressed and present in lower molecular weight forms in 18/20 tissue samples which is consistent with the role of cyclin E as a prognosticator for breast cancer (73, 74, 80). The pattern of cyclin E expression observed in these tumor specimen were similar to those used in a previous study (50) showing presence of lower molecular weight forms of cyclin E with increasing stage of the disease. Interestingly most of the tumor specimen which showed an overexpression of cyclin E also were negative for ER and PR. A negative steroid receptor status is indicative of poor response to endocrine and cytotoxic chemotherapy characteristics of very aggressive breast tumors (81). Furthermore p16 was overexpressed in 7 (i.e KK-005, 086, 147, 173, 190, 369, and 399) out of the 20 samples examined. Three of these 7 samples had a defect in pRb expression, while in the remaining 4 samples (i.e KK-005, 147, 173, and 369) pRb was expressed and present in multiple bands, suggesting a functional protein. In addition cyclin E was severely overexpressed in all 4 p16/pRb double positive samples. Hence, these observations suggest that *in vivo*, in breast cancer tissues which overexpress cyclin E, overexpression of p16 is not always accompanied by a defect in pRb, consistent with results obtained with MDA-MB-157 cell line. Cyclin E which is overexpressed and present in lower molecular weight forms in these tumor tissue samples may be capable of

phosphorylating pRb in the absence of functional cyclin D containing complexes *in vivo* as well as in cell lines.

Cyclin E is present in E2F complexes throughout the cell cycle of tumor but not normal cells. One of the major targets of growth regulation by pRB is the E2F family of transcription factors. During the G1 phase of the cell cycle, underphosphorylated pRB binds to E2F and represses its transcriptional activity. Phosphorylation of pRB by cyclins during late G1 and S phase release E2F, that in turn leads to activation of the transcription of genes important for cell cycle progression. Similarly, p107 and p130, two pRB-related proteins, regulate the transcriptional activity of E2F. In addition, both cyclin A and E can bind to p107 and p130 while in complex with E2F. While the significance of this association is not known, it has been suggested that it regulate the transcriptional activity of E2F.

To determine, whether the cyclin E overexpression in the tumor cell lines affected the E2F DNA binding complexes throughout the cell cycle, we performed bandshift assays using an oligonucleotide with an E2F binding site as a probe. As a control, extracts from a synchronized population of normal cells were prepared. As described previously (82), normal cells contained several E2F complexes that were present at various times in the cell cycle. The disappearance of E2F complexes at 6, 9 and 12 hours after release from the thymidine block occurred when the cells were enriched for G2/M (82) (figure 3A). The complex marked with an arrow contained the pRB-related protein p107 and cyclin A, as shown by antibody supershift analysis (data not shown). Addition of cyclin E antibody did not have any effect on the mobility of this complex (figure 3A), suggesting that cyclin E is not the predominant cyclin in the p107/E2F complex in normal cells. On the other hand, in extracts prepared from tumor cells, E2F complexes were present throughout the cell cycle and no loss of these complexes was observed during G2/M. The complex marked with an arrow could be disturbed with anti-p107 and partially with anti-cyclin A antibodies (data not shown). The addition of an anti-cyclin E antibody resulted in a super shift of a large proportion of the complex, suggesting that most of the p107-E2F complex contained cyclin E

(figure 3B). Addition of antibodies to cyclin A and cyclin E to the same extract did not result in the appearance of any different complex than when both antibodies were added independently (data not shown), suggesting that both cyclins did not form part of the same complex. The association of cyclin E with the E2F complexes in tumor cells paralleled the constitutive expression of cyclin E throughout the cell cycle (Fig 2A, right panel). Hence, overexpression of cyclin E in tumor cells was capable of forming a major complex with p107 and E2F. This is a second example of how overexpression and constitutive expression of cyclin E could result in a dual role for this cyclin allowing redundancy in function.

CKIs are differentially expressed in normal versus tumor-derived exponentially growing cells. In order to determine the relative levels of CKIs in normal versus tumor-derived breast epithelial cells, we initiated our studies by investigating the expression of four CKIs (p21, p27, p15 and p16) in three normal cell strains, one immortalized normal cell line and nine mammary epithelial tumor cell lines (Fig 4). Table 1 summarizes the mammary epithelial cell types, estrogen receptor (ER), p53, pRb (retinoblastoma), and cyclin E status of these cells. Total RNA and protein were prepared from exponentially growing cells and subjected to Northern or Western blot analyses. As demonstrated, p21 mRNA is expressed at higher levels in normal versus tumor cells; whereas, p27 and p16 mRNAs are more highly expressed in tumor cells (Fig. 4A). This pattern of expression is also evident at the protein level as demonstrated by Western blot analysis (with the exception of p15 which is overexpressed in normal cells at the level of protein, but not mRNA-see below) (Fig. 4B). These observations suggest that *in vivo*, within a particular cell type, the CKIs may have different functions; specifically p16 and p27 overexpression/alteration may represent a gain of function phenotype which may endow tumor cells with a growth advantage while p21 and p15 may function as tumor suppressors. Although the levels of mRNA for each of the CKIs generally correspond to their protein levels, some particular discrepancies are noted with respect to p15 expression. In the normal cell strains the p15 protein is expressed at high levels; whereas, mRNA levels are only highly expressed in the 81N

cells. Additionally, the tumor cell line Hs-578T expresses very high levels of p15 mRNA and undetectable levels of p15 protein. These observations suggest that p15 levels may be regulated by post-transcriptional, translational or post-translational mechanisms.

Expression of CKIs in synchronized normal and tumor cells.: The CKIs have been proposed to establish a threshold of inhibition which must be exceeded if cell cycle progression is to occur (83). Therefore, any disruption in the levels of CKIs or cyclin-cdks could offset the threshold balance or result in the displacement of particular regulatory proteins. The net result of such an imbalance might be aberrant cell cycle progression. This led us to analyze the pattern of expression and relative levels of these CKIs throughout the cell cycle in synchronized populations of both normal cell strains and tumor cell lines (Figs 5-7).

Initially we assessed the effect of growth factors on CKI expression by arresting normal 76N cells in G0 (Fig 2) (i.e. growth factor deprivation induced quiescence). This allowed us to determine if a particular phase of the cell cycle might enrich for the expression of these CKIs. Briefly, cells were cultured in growth factor deficient medium for 72 hours and then stimulated to re-enter the cell cycle with addition of growth factors. Re-entry into the cell cycle and S phase was monitored by [^{3}H] thymidine incorporation (Fig. 5B). At various times after re-addition of growth factors, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to p21, p27, p15, p16 and cyclin A (Fig 5A). In normal 76N cells, the pattern of expression of cyclin A protein is consistent with that seen for other normal cell types with levels rising dramatically at S phase and disappearing by the end of G2/M phase. The pattern of expression of the 4 CKIs examined following growth factor stimulation indicates that p21 levels increase substantially (10 fold) during S phase, while p27, p15, and p16 levels remain relatively unchanged throughout the cell cycle (Fig. 5A). p16 levels are very low, and a much longer exposure time (i.e. 10 min for p16 versus 10-30 sec. for the other CKIs) was required to detect the expression of this protein in 76N cells. These observations again corroborate our hypothesis that these CKIs may function differently from each other during the cell cycle, with p21 most likely

having an essential role during S phase of cycling cells and p27 and p15 being more important in quiescent cells.

Induction of p21$^{CIP1/WAF1}$ and p27^{KIP1} by Lovastatin in tumor cells. In order to synchronize tumor cells in the G1 phase of the cell cycle, MDA-MB-157 breast cancer cells were treated with Lovastatin, an inhibitor of the cholesterol biosynthetic pathway (Fig 6). (We have previously reported on the use of Lovastatin as an agent to synchronize cells in early G1 (84, 85)). Cells were cultured in Lovastatin for 36 hours at which time Lovastatin media was removed and replaced with media containing Mevalonate (the end product of the cholesterol biosynthetic pathway), which is routinely used to stimulate cells to re-enter the cell cycle and advance to S phase. Synchrony of tumor cells at various times after release from the Lovastatin block was monitored by [^{3}H] thymidine incorporation (Fig 6E). Immediately following Lovastatin treatment there is inhibition of DNA synthesis followed by a dramatic increase, indicative of the cells being arrested in the G1 phase of the cell cycle.

Total RNA and protein were extracted from cells harvested at various times after release from Lovastatin and analyzed on Northern and Western blots using CIP1/WAF1 and KIP1 cDNAs and p21 and p27 antisera as probes (Fig 6A-B). Unexpectedly, we found that Lovastatin treatment dramatically induced the expression of p21 and p27 in MDA-MB-157. These 2 CKIs are barely detectable in exponentially growing tumor cells (Fig 4B). The Lovastatin induction is evident at both the transcriptional (p21) and translational (p21 and p27) levels as demonstrated by Northern (Fig 6A) and Western blot (Fig 6B) analyses. Although the CKI levels were substantially induced for a few hours, the levels abruptly diminished. In order to determine whether the transient induction of the CKIs led to the inhibition of kinase activity, we measured the phosphorylation of histone H1 in anti-cdk2 immunoprecipitates prepared from extracts of synchronized cells (Fig 6C). These results indicate that the induced levels of p21 and p27 are accompanied by a dramatic decrease in cdk2 associated kinase activity which then increases upon disappearance of p21 and p27 proteins. In order to determine if p21 and/or p27 can directly

associate with cdk2 and inhibit its activity, we performed a 2 step immunoprecipitation/Western blot analysis where we immunoprecipitated with anti-cdk2 antisera followed by immunoblotting with p21 or p27 antisera (Fig 6D). Anti-p21 immunoblot analysis of cdk2 containing complexes demonstrates that p21 is directly associated with cdk2 and that the relative amount of p21 associated with cdk2 is inversely correlated with kinase activity of cdk2.

p27 is also similarly associated with cdk2 although in relatively less abundance than p21. Together these analyses indicate that not only are p21 and p27 induced during Lovastatin synchronization in MDA-MB-157 tumor cells, but they functionally complex with cdk2. Furthermore the expression of p21 and p27 is inversely correlated with cdk2 kinase activity suggesting that these CKIs may directly inhibit cdk2.

Induction of p21 and p27 in tumor cells by Lovastatin is cell cycle independent: To investigate whether the Lovastatin mediated induction of p21 and p27 is cell cycle dependent or due to a direct (as of yet unknown) effect of Lovastatin, and to compare the cell cycle pattern of expression of the 4 CKIs in normal and tumor cells, we synchronized both normal and tumor cell types in the G1/S boundary by double thymidine block (Fig 7). Synchrony of both cell types after release from block was monitored by flow cytometry (Fig 7C). At various times after release from treatment, cells were harvested and extracted proteins were analyzed on Western blots with antisera to p21, p27, p16, p15 and cyclin A. In both normal and tumor cells, the pattern of expression of cyclin A protein is consistent with that seen for other cell types with levels being very tightly regulated such that peak levels occur during S and early M phase (Fig 7A).

The pattern of expression of p21 in normal cells revealed a very tight cell cycle regulation and the results confirm our growth factor deprivation results of figure 2 indicating that in normal cells p21 is cell cycle regulated with peak levels coinciding with peak S and early M phase (Fig 7A, left panels). In tumor cells, however, p21 levels remain low and virtually undetectable during all phases of the cell cycle. Hence, we were not able to enrich for p21 expression at the G1 phase of tumor cells synchronized by double thymidine block (Fig 7B, right panels). These observations

strongly suggest that the induction of p21 during Lovastatin synchronization (Fig 7) is not cell cycle dependent but is rather a Lovastatin or mevalonate specific effect.

The level of p27 remained unchanged in both normal and tumor cells synchronized by double thymidine block. Similar to p21, p27 levels were not further induced in the G1 phase of tumor cells synchronized by double thymidine block, suggesting that the induction of p27 during Lovastatin synchronization (Fig 6B) is not a cell cycle dependent effect, but rather due to Lovastatin effect. p16 levels were only minimally and constituitively detected in normal cells (detection was possible only upon prolonged exposure times (i.e. 10 minutes versus 10-30 sec for the other CKIs), and in tumor cells p16 was constituitively overexpressed throughout the cell cycle. p15 was expressed throughout the normal cell cycle and was undetectable in tumor cells even upon very long exposure of the Western blots (i.e. 24 hrs).

In order to compare the kinase activity associated with cdk2 in normal and tumor cells, we measured the phosphorylation of histone H1 in anti-cdk2 immunoprecipitates prepared from synchronous cell extracts (Fig 7B).There was a significant difference between normal and tumor cells in the timing of cdk2 activity. In normal cells, the cdk2 associated kinase activity is cell cycle regulated, coinciding with the increased levels of cyclins E (86) and A protein expression (Fig 7A). Conversely, in tumor cells cdk2 remains catalytically active throughout the cell cycle resulting in a constitutive pattern of histone H1 phosphorylation (Fig 7B). Therefore, the observed decrease in cdk2 associated kinase activity in Lovastatin treated MDA-MB-157 cells (Fig 6C) is most likely a direct result of inhibition mediated by the induction p21 and p27 proteins by Lovastatin.

Kinetics of induction of CKIs in normal and tumor cells by Lovastatin: We next analyzed the kinetics of expression of the four CKIs (p21, p27, p15 and p16) in Lovastatin treated MDA-MB-157 tumor and 76N normal cells (Fig 8). Cells were treated with Lovastatin for 36 hours, at which time they were washed and incubated with fresh medium containing mevalonate. At various times (6 hr intervals) during the Lovastatin/Mevalonate treatments, cells were harvested

and extracted protein and RNA were analyzed on Northern and Western blots with different CKI cDNAs and antisera as probes (Fig 8).

Western blot analysis revealed that in both normal and tumor cells either one or all the CKIs were induced (Fig 8A). Interestingly, we observed an induction of p16 protein in normal cells and an induction of all four CKIs in tumor cells (both protein and mRNA). Perhaps the most intriguing observation is the kinetics of the induction of the inhibitors. The induction of p16 protein in normal cells occurs specifically at 30 hour post Lovastatin treatment. Until this time p21, p27 and p15 protein levels remain relatively unchanged, but at 30 hours these proteins abruptly disappear (Fig 8A, left panel). The induction of p21, p27 and p16 proteins in tumor cells occurs approximately 18 hours post Lovastatin addition and levels progressively accumulate until 30 hours (Fig 8A, right panel). At this time p15 protein, which is usually not expressed in tumor cells, is induced to very high levels while levels of p21 and p16 abruptly diminish. Following the disappearance of p15, both p16 and p21 dramatically reappear for the next few time intervals examined, demonstrating a bi-phasic pattern of expression as a result of Lovastatin treatment.

The induction of p21, p27, and p16 in tumor cells correlates with an induction of their mRNAs (Fig 8B). However, the disappearance of these proteins is a post-transcriptional event as mRNA levels remain high at 30 hours, and do not further accumulate at subsequent time intervals (Fig 8B). There is also a discrepancy in the length of time which p15 mRNA versus protein is induced by Lovastatin. p15 mRNA levels in tumor cells accumulate at 6 hours post Lovastatin treatment and starts to disappear 30 hrs later (i.e. 6 hours post mevalonate treatment). However, p15 protein is only induced at one time interval (30 hr) by Lovastatin treatment (Fig 8A). Similarly, in normal cells, p21 and p27 mRNAs are both induced early on by Lovastatin and do not diminish significantly during the course of treatment. Furthermore, p15 mRNA is present at very low levels during the early hours of Lovastatin treatment of normal cells and is significantly induced at 30 hours (Fig 8B). Lastly, the abrupt induction and subsequent disappearance of p16 protein in normal cells is also due to post-transcriptional mechanisms as p16 mRNA levels remained

constituitively high during the 36 hours of Lovastatin treatment. Collectively these data suggest that treatment of cells by Lovastatin leads to the induction of CKIs in a cell cycle independent fashion. Furthermore, this induction results in a concerted and cooperative interaction between the two families of the CKIs which could then lead to the orderly withdrawal of the cells from the cell cycle.

Lastly, in a new study to delineate the mechanisms of alteration of cyclin E, we asked the question whether within a tumor cell line, there are subpopulations of cells which express different forms of cyclin E. For that purpose we chose MDA-MB-157 cell line which as our data depicts contains different forms of cyclin E. We subcloned this cell line into several clones by serial dilution. We obtained over 40 clones and have initiated our analysis on these clones. One of our analysis is the determination of growth kinetics of each of these clones and correlating the doubling times of the subclones to cyclin E levels. As depicted in figure 9, the doubling times of the subclones ranged from 45 to 74 hours. Our preliminary data on cyclin E levels in these cells shows that as the doubling time of the subclones increases, so does the appearance of lower molecular weight isoforms of cyclin E. In faster growing subclones, only the higher molecular weight isoforms of cyclin E were observed. These results suggest that there may be a cause and effect relationship between cyclin E alteration and growth kinetics of cells. We will pursue these studies, which are a part of task 4, in years 3 and 4 of our application.

7: Conclusions/Discussion:

The first Aim of our studies, use of cyclin E antibody as a diagnostic prognostic marker for breast cancer is an ongoing one. However, we have surpassed our initial goal of collecting and extracting 150 tissue samples per year by increasing this number to 550 samples. We also have completed our analysis of cyclin E and other tumor markers by Western blot analysis and have obtained all the pertinent clinical information on each patient and are in the process of correlating cyclin E alteration to patient outcome. We are working toward completion this aim by the third year

of this grant application as originally anticipated and hence do not anticipate any major changes or problems.

The second Aim of the application deals with utilizing the deletional mutations of cyclin E to detect early metastatic breast cancer. We have documented (86) that these truncated forms of cyclin E are not deletional mutations and are in fact splicing variants of cyclin E found in normal and tumor cells and tissue samples. As outlined in the third aim of our application we have also investigated the regulation of cyclin E in normal versus tumor cells and found that cyclin E is deregulated in tumor cells. Such deregulation of cyclin E in tumor cells leads to its functional redundancy. Lastly, we have initiated our studies on the oncogenic potential of cyclin E in normal cells and its interplay with p21 and other cyclin-dependent kinase inhibitors. Below is a discussion of the results presented in this report.

The interplay between cyclin D1/cdk4-cdk6/p16/pRB has been implicated as a crucial G1-phase controlling pathway which becomes frequently de-regulated in many types of cancer. Any mutations which could give rise to an imbalance in any one of these cell cycle regulatory proteins may therefore result in a cell growth advantage and eventually lead to tumorigenesis. In this model overexpression of p16, which specifically interferes with the cyclin D-dependent kinases cdk4 and cdk6, prevents cdk4/cdk6 from phosphorylating pRb, and this functional inactivation of pRb leads to a G1 block (87-89). Thus, p16 is thought to negatively regulate the cell cycle (46). In fact, several studies have documented that primary tumors which showed expression of functional pRb protein did not express p16 protein (due to mutations in the gene) and conversely, cells that expressed p16 protein, did not have a detectable pRb protein (90-93). These studies collectively suggest that there is a linkage between the expression and function of D type cyclins, cdk4/cdk6, pRb and p16, such that overexpression of cyclin D1, inactivation of pRb or loss of p16 may have equivalent consequences for loss of normal growth control. In addition, this model predicts a lack of functional redundancy of this pathway with other cell cycle regulatory proteins.

Even though many studies have corroborated the p16/pRb inverse correlation model, there have also been documentation to the contrary. For example in their analysis of pRb and p16

expression in lung cancers, Otterson et al.(92) reported that 14% of Small Cell Lung Cancers and 15% of Non-Small Cell Lung Cancers (NSCLC) examined were p16 and pRb double positives, and Sakaguchi et al. (94) reported that 16.4 % of NSCLC studied immunohistochemically also stained positively for both p16 and Rb protein. In addition Gerardts et al. (95) who developed an immunohistochemical assay used to assess the expression of both p16 and pRb in formalin-fixed, paraffin-embedded tissues report that in 43% of all carcinomas examined (i.e. breast 5/20, bladder 7/19, colon 16/19, and lung 4/17), both pRb and p16 could be detected suggesting that in common human malignancies p16 and pRb expression is not mutually exclusive. Furthermore, Musgrove et al. (96) report that in 50% (i.e 10/20) of breast cancer cell lines examined INK4p16 mRNA was expressed in the absence of any pRb mutations. Lastly Ueki et al. (97) show that 13% of glioblastoma cell lines examined showed neither p16 nor RB alterations and Wang et al. (98) studying human melanoma cell lines report that regardless of the status of p16 protein, all 15 melanoma cell lines examined showed the presence of pRb protein ruling out an inverse correlation between the expression of p16 and pRb in these particular cell lines.

One possible explanation for the lack of inverse correlation between p16 and pRb may be due to overexpression of cyclin E in these cell lines and tumor samples which could act redundantly in function and replace cyclin D/cdk complexes for phosphorylating pRb. In concordance with this redundancy hypothesis Hinds et al. (63) first demonstrated that overexpression of several different cyclins, including cyclin E, could override the growth arrest properties of pRB in SaOS-2 cells. In addition we had previously reported that cyclin E is severely overexpressed in all breast cancer cell lines examined (73). The overexpression of cyclin E and expression of its lower molecular weight forms not only were evident in tumor tissue specimen obtained from several different malignancies including breast carcinoma, but they can also be used prognostically for breast cancer (74, 80). Recently, we also showed that overexpression of cyclin E is accompanied by its constitutive expression and activity throughout the tumor cell cycle (86). Since cyclin E is overexpressed and forms a complex with cdk2 constituitively, the active complex can act upstream of pRb and phosphorylate it even when cyclin D is inactive due to overexpression

of p16. To test this model, in this study we used a breast cancer cell line which exemplified an exception to the inverse correlation rule of p16/pRb. In this tumor cell line (i.e. MDA-MB-157) cyclin E is markedly overexpressed and present in lower molecular weight isoforms, p16 is also overexpressed and pRb is not mutated and detectable in both its hypo- and hyperphosphorylated forms. Under these conditions we show that p16 binds to both cdk4 and cdk6 and inhibits the binding of cyclin D1 to these cdks. We also provide evidence that in synchronized populations of MDA-MB-157 cells pRb is phosphorylated through out the cell cycle following an initial lag revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. These analysis suggest that cyclin E/cdk2 and not cyclin D/cdk4-cdk6 is a candidate kinase complex capable of phosphorylating pRb through out the cell cycle of this tumor cell line.

To directly examine the lack of inverse correlation of p16 and pRb in vivo we document in Table 2 that in breast tumor specimen obtained from breast cancer patients diagnosed with end stage disease where cyclin E is markedly overexpressed, and p16 is also overexpressed, pRb is detectable in both its hypo and hyperphosphorylated forms. These studies suggest that phosphorylation of pRb under conditions were cyclin D/cdk complexes are rendered inactive is not an artifact of the culture conditions and occurs in vivo.

Since cyclin E is constituitively expressed in MDA-MB-157 cancer cells, and is present during times in the cell cycle when cyclin A is not detected (see figure 2), it followed that cyclin E could also replace cyclin A containing complexes. In fact as displayed in Figure 3 cyclin E can function redundantly and replace cyclin A in E2F complexes with cdk2 and p107 in tumor cells. In normal cells, cyclin E was found in complex with the pRB-related proteins p107 and p130 and E2F during the late G1 and early S phase of the cell cycle. We have found that while this cyclin was a minor component of E2F DNA binding complexes in normal cells, it was a major component of this complex in MDA-MB-157 cells. Interestingly, while normal cells display a down regulation of E2F DNA binding activity in the G2/M phases of the cell cycle, MDA-MB-157 cells show constitutive E2F DNA binding complexes through the cell cycle. This raises the

possibility that overexpression of cyclin E perturbed the regulation of E2F activity not only by promoting the hyperphosphorylation of pRB but also by perturbing the cell cycle regulation of E2F by p107.

Based on our observations in breast cancer cell lines and tumor tissue samples we suggest an alternative order of events along the G1 phase controlling pathway culminating in phosphorylation of pRB. In this pathway cyclin E would act upstream of pRb bypassing cyclin D/cdk4 and giving the tumor cells a selective growth advantage even in the presence of high levels of p16. Hence abrogation of cyclin D1, cdk4/cdk6 or p16 will not have any affect on the phosphorylation of pRb which will be accomplished by cyclin E/cdk2 in these cells leading to a deregulated progression through G1. Our data also demonstrates that cyclin D1 is not required for G1 progression in tumor cells which exhibit an overexpressed cyclin E and a wild-type pRB. As a result the function of cyclin D1 is dispensable not only in cell lines in which pRb is inactivated as described (99), but also in cell lines were cyclin E is overexpressed and constituitively active (this study and (65). Lastly, this study provides evidence for a lack of functional link between p16 and pRb suggesting that in sub populations of breast cancers pRB is not a major substrate for the inhibitory activity of the p16 product. Hence certain populations of tumor cells can overcome the role of p16 as a tumor suppressor protein by providing a redundant pathway to inactivate pRB and provide a growth advantage to the cells.

The studies reported here also represent a comprehensive analysis of the expression of four CKI mRNAs and proteins including both the $p21^{CIP1/WAF1}$ and Ink4 families in normal and tumor mammary epithelial cells. Our data indicate that striking differences exist amongst the individual CKIs between normal and tumor cells with p21 and p15 expression higher in normal cells and p27 and p16 more abundantly expressed in tumor cells. Additionally, the cell cycle expression levels of the CKI proteins are different suggesting that these inhibitors may be functionally distinct throughout the cell cycle and that p21 and p15 may possess growth inhibitory activities whereas p27 and p16 may provide tumor cells with a growth promoting advantage. We have found that the

drug Lovastatin is capable of inducing all four CKIs in a cell specific manner via a cell cycle independent mechanism. We also show that the regulation of CKIs is subject to exquisite regulation both temporally and with respect to their relative levels. The unusual kinetics of CKI induction strongly suggest a cooperativity in CKI regulation and/or function. Lastly, comparative analysis of CKI proteins and mRNAs indicate that they are regulated by both transcriptional (induction) and post-transcriptional mechanisms (their sudden disappearance at 30 hours post Lovastatin addition).

We propose that the induction of the CKIs by Lovastatin is through cell cycle independent mechanisms. The mechanism by which Lovastatin synchronizes cells is still unknown. Lovastatin, an inhibitor of HMG CoA reductase (the first enzyme in the isoprenyl lipid biosynthetic pathway), is a widely used drug for the treatment of hypercholesterolemia. Lovastatin prevents the first step of cholesterol synthesis, which is the conversion of HMG into mevalonic acid. The blockage of this pathway also prevents the isoprenylation of several proteins such as Ras, Rap and G proteins by farnesyl, a downstream product of the pathway. This inhibition of isoprenylation blocks the function of these proteins (100, 101). Apart from its inhibitory action on HMG CoA reductase, Lovastatin has also been used as an effective agent in cell synchronization for both tumor and normal cells (84, 85). Recently Hengst et al (102, 103) reported an elevation of p27 in Lovastatin arrested HeLa cells. This increase was presumably attributed to a cell cycle effect as a similar increase was observed in cells synchronized by density mediated arrest, thymidine and nocodazole blocks (102). It is imperative to note however that in this study the increase in p27, in density mediated arrested and thymidine blocked cells, was much lower than Lovastatin treated cells. The authors in this study related a persistent p27 expression in density mediated and thymidine blocked cells to imperfect synchronization of the cells, indicating that the increases seen might be due to experimental setup. Furthermore HeLa cells, transformed by the human adeno papilloma virus, do not represent a general human tumor cell line. In this report, we show that even though Lovastatin is capable of inducing both p21 and p27 in human breast tumor cells, this induction is not due to cell cycle synchronization affects of Lovastatin. The induction of

the inhibitors was not observed by other methods of cell synchronization such as double thymidine block in normal and tumor cells or growth factor deprivation in normal cells. Furthermore, we used both normal and tumor cells which were derived from human mammary epithelial cells, and since over 90% of all human tumors are of epithelial origin, the cells used in this study are representative of most tumor cell lines. Hence even though Lovastatin is capable of inducing these inhibitors in human epithelial cells, the mechanism of induction is not through arrest of cells in a specific phase of the cell cycle, but through a Lovastatin, drug, mediated affect. Whether the CKI induction following Lovastatin treatment is due to inhibition of any one reaction of the cholesterol biosynthesis pathway, it remains to be elucidated.

Apart from increases in p21 and p27, Lovastatin also induced expression of p16 and p15. The expression of these INK4 kinase inhibitors were biphasic, time and cell line specific. In normal cells following 30 hours of Lovastatin treatment, a significant and transient induction of p16 was observed, whereas the other CKI's levels remained unchanged up to 30 hours but fell rapidly following 30 hours of treatment. These observation suggest that p16 expression may initiate a feedback loop which inhibits the expression of the other kinase inhibitors in normal cells. Tumor cells showed similar pattern of biphasic induction of the kinase inhibitors except that p21, p16, and p27 expression are induced after 18 hours and continue to increase up to 30 hours, at which time p15 is expressed for the first time. Simultaneously with p15 expression, the levels of p21 and p16 rapidly diminish, only to reappear once p15 expression has abated. This pattern of expression of p15 in tumor cells and p16 in normal cells follows a "window phenomenon", such that the expression of these kinases inhibitor is short and dependent on the time following induction. The kinetics of expression of these kinases strongly suggest a cooperative mechanism between the two families of CKIs which results in growth arrests.

Similar cooperative interactions was demonstrated between p27 and p15 in MvlLu cells treated with cytokine transforming growth factor ß (TGF-ß) (104). Following treatment with TGFß the expression of p15 is elevated and corresponds to the release of p27 from cdk4 and cdk6. This release from cdk4 enables p27 to bind to cdk2 and inhibit its activity. Similarly, p16

elevation was seen in transformed fibroblast cells followed by dissociation of cyclin D-cdk4-p21 binding complexes (29). This dissociation allows p16 binding to cdk4 and its subsequent inactivity. These studies, taken together with those reported here suggest that CKIs are extremely dynamic proteins whose levels are closely monitored and adjusted in response to changing of environmental conditions. The cellular responses to alterations in CKI levels most likely involve positive and negative feedback loops and result in extremely rapid and complete elimination of distinct CKIs. A likely mechanism for the rapid disappearance of these proteins would be the ubiquitin dependent proteolytic system which has been demonstrated to regulate p27 levels (105).

In the studies reported here, we simultaneously examined all 4 CKIs at the level of transcription and translation and observed a complex and coordinate regulation of CKI mRNAs and proteins in Lovastatin treated breast cells. Our findings strongly suggest that distinct yet coordinately regulated pathways control the expression of the two divergent groups of CKIs in both normal and tumor cell types. The kinetics of CKIs expression following Lovastatin treatment suggest that both families of mammalian CDK inhibitors participate cooperatively in the growth inhibitory mechanism of the cell. This regulation involves both transcriptional and post-transcriptional mechanisms.

The abrupt appearance of a particular CKI (p16 in normal and p15 in tumor cells) and coincident disappearance of other CKIs suggest that these proteins may act antagonistically in each other's regulation. p16 and p15 may act as "master molecules" and initiate a pathway by which the transcriptional expression of the other CKIs is repressed or their proteins degraded. Alternatively, p15 and p16 may be induced to compensate for the loss of the other CKIs. While the overall expression of CKIs varies between normal and tumor cells suggesting their deregulation, vestiges of the regulatory pathway remain intact and capable of cross talk. The altered expression of CKIs in tumor cells may be key to their tumorigenic phenotype while their regulation by Lovastatin may provide a novel therapeutic approach to cancer treatment. In the very least these analyses provide a model system for the comparative analysis of transcriptional and post-transcriptional regulation of CKIs in both normal and tumor cells.

8. References

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9. Appendix

The figure and figure legendss referred to in the text are presented in this section.

We are also including a copy of 4 manuscripts (published and/or in press) summarizing the results in this annual report.

Table 1: Characterization of normal and tumor-derived breast epithelial cells

Cell Lines	Cell Types	Estrogen Receptor (73)	pRb*	P53	Cyclin E (73, 86)
1 - 70N	N-mortal	-	+	+ (106)	+
2 - 81N	N-mortal	-	+	+ (106)	+
3 - 76N	N-mortal	-	+	+ (106)	+
4 - MCF-10-A	N-immortalized	-	+	+ (107)	+
5 - MCF-7	A (pe)	+	+ (109)	+ (107)	+++
6 - MDA-MB-157	C (pe)	-	+ (75)	- (107)	++++++
7 - MDA-MB-231	A (pe)	-	+	- (107)	++++
8 - MDA-MB-436	A	-	_ (75)	- (108)	+++++
9 - T47D	DC (pe)	+	+ (109)	- (107)	++
10 -BT20	C	+	+	+ (107)	++
11 - HBL-100	T (bm)SV40 transformed	-	-	- (107)	+++
12 - HS-578T	DC	-	-	- (107)	++++
13 - ZR75T	IDC	+	+	+ (107)	+++

N, normal breast cells from reduction mammoplasty; A, adenocarcinoma; pe, pleural effusion; C, carcinoma; DC, ductal carcinoma; T(bm), tumor breast milk); IDC, infiltrating DC. Cell type, Estrogen receptor (ER), p53 and Cyclin E status as determined in indicated references. + indicates wild type, ++(++++) indicates various degrees of overexpression with MDA-MB-157 showing the highest degree of cyclin E overexpression, - mutant or not expressed. *pRb data is obtained though Western blot analysis using a monoclonal antibody against pRb (PharMingen, San Diego, CA)

Table 2: Correlation of p16 and pRb status in a series of breast carcinomas

Patient ID #	ER/PR[1]	DNA Index/ Ploidy[1]	Proliferation Index (%)[1]	Cyclin E[2]	p16[2]	pRb[2]
KK005	-/-	1.18/Aneuploid	12.2 (H)	+++	++++	+
KK017	-/-	1.72/Aneuploid	1.5 (L)	++++++	±	+
KK020	-/-	1.73/Aneuploid	14.1 (H)	+++++	-	-
KK036	+/-	1.84/Tetraploid	3.3 (L)	++	±	+
KK061	-/-	ND	ND	++++	±	-
KK070	+/+	ND	ND	+	±	-
KK076	-/-	2.08/Tetraploid	12.5 (H)	+++	±	-
KK086	-/-	1.50/Aneuploid	36.0 (H)	+++++	++	-
KK147	ND	ND	ND	+++++	+++	+
KK173	+/-	1.91/Tetraploid	30.2 (H)	+++++++	++++	+
KK190	-/-	2.09/Tetraploid	31.8 (H)	+++++++	+++	-
KK322	+/-	2.70/Aneuploid	30.0 (H)	+++	-	+
KK369	ND	ND	40.0 (H)	+++++++	++++	+
KK399	-/-	ND	ND	++++	++++	-
KK400	+/-	ND	ND	++++	±	-
KK407	-/-	1.89/Tetraploid	18.0 (H)	++++	-	-
KK428	-/-	1.75/Aneuploid	27.0 (H)	++++	-	-
KK429	-/-	1.71/Aneuploid	28.0 (H)	+++++	-	-
KK457	ND	ND	ND	++++++	-	+
KK458	-/-	1.96/Tetraploid	11.3 (H)	+	-	+

1: Quantitation of immunohistochemical staining by image analysis was performed on sections stained with either the monoclonal antibody to Estrogen Receptor, H222 (ER-ICA Kit, Abbott Laboratories, North Chicago, IL), monoclonal antibody to Progesterone receptor, mPRI, (Cell Analysis Systems, Inc,. Lombard, IL) or monoclonal antibody to Ki67 (DAKO-PC, Dako Corporation, Santa Barbara CA) as described (34, 35). KI67 staining determined growth fraction of the tumor. Values indicate percent positive staining: 1.0-7.0% is indicative of low (L) proliferation index, 7.1-11.9 is indicative of moderate (M) proliferation index and >12.0% is indicative of high (H) proliferation index. For each case the DNA ploidy was determined by quantitation of the DNA Feulgen stain by computerized microdensitometry as described (58).

2: Cyclin E, p16, and pRb levels were measured using Western blot analysis with HE12 monoclonal antibody to cyclin E (Santa Cruz Biotechnology, San Diego, CA) as described (38, 40), monoclonal antibodies to p16 and pRb as described in text. Levels of cyclin E in tumor tissue samples were correlated with 76N normal (+) and MDA-MB-157 (++++++) tumor cell lines. For example, cyclin E in MDA-MB-157 cell line is 64 fold (i.e. ++++++) overexpressed compared to 76N cell line (i.e. +) (38). Any tumor tissue overexpressing cyclin E more than MDA-MB-157 received 7 +s (i.e +++++++). P16 levels were also correlated with MDA-MB-157 (++++) cell line. Equal protein loading was monitored by reprobing blots with actin and all blots were analyzed by densitometry using AGFA scanner and IP Lab Gel software.

Figure Legends:

Figure 1: Expression and complex formation of p16/pRB pathway proteins in normal and tumor derived breast epithelial cells. **(A)** Western blot analysis: Exponentially growing normal and tumor cells were subjected to Western blot analysis using 50µg protein for each cell line in each lane of either a 6% (pRb) 13% (cyclin D1, cdk4, and cdk6), or 15% (p16) acrylamide gel and blotted as described. The same blot was reacted with cyclin D1, cdk4 and cdk6 affinity purified antibodies. The blots were stripped between the three antibodies in 100 mM ß-mercaptoethanol, 62.5 mM Tris HCL (pH 6.8) and 2% SDS for 30 min at 55 °C. **(B)** Immune-complex formation: For immunoprecipitation followed by Western blot analysis equal amounts of protein (500 µg) from cell lysates prepared form each cell line were immunoprecipitated with either monoclonal antibody to p16 (p16/cdk4 and p16/cdk6), polyclonal antibody to cyclin D1 (cyclin D1/cdk4) or a monoclonal antibody to cyclin D1 (cyclin D1/cdk6), coupled to protein A/G beads and the immunoprecipitates were washed, boiled for 3 min, separated by SDS-13% PAGE, blotted to Immobilon membranes, and hybridized with either polyclonal antibody to cdk4 (p16/cdk4), polyclonal antibody to cdk6 (p16/cdk6 and cyclin D1/cdk6-arrow pointing to the complexed protein), or monoclonal antibody to cdk4 (cyclin D1/cdk4). The list of normal and tumor cell lines is presented in Table 1 using identical numbers.

Figure 2: Phosphorylation of pRb in synchronized population of tumor versus normal cells. Both cell types were synchronized by double thymidine block procedure (see Materials and Methods). At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to **(A)** Western blot and **(B)** Histone H1 or GST-Rb kinase analysis. Protein (50µg) for each time point was applied to each lane of either a 6% (pRB) or 10% (cyclins E and A) acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity purified polyclonal antibodies. The blots were stripped between the two assays as described for figure 1. For kinase activity, equal amount of proteins (600 ug) from cell lysates prepared from each cell line at the indicated times were

immunoprecipitated with anti-cyclin E (polyclonal) coupled to protein A beads using either histone H1 or purified GST-Rb as substrates. **(C)**: The relative percentage of cells in different phases of the cell cycle for each cell line at various times after release from double thymidine block was calculated from flow cytometric measurements of DNA content. (◆) cells in S phase, (o) cells in G2/M phase, and (❑) cells in G1 phase.

Figure 3: Cyclin E is the predominant cyclin in p107/E2F complexes in tumor cells: E2F complex were analyzed by gel retardation assays using cell lysates (15μg) prepared from synchronized populations (see figure 2) of **(A)** normal 76N and **(B)** of tumor MDA-MB-157 cells. The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human DHFR promoter. 200ng of the anti-cyclin E antibody was used to disrupt the E2F complexes.

Figure 4. Altered Expression of CKIs in exponentially growing normal Versus tumor cells.

Northern blot **(A)** and Western Blot **(B)** analyses of CKI expression in normal versus tumor breast epithelial cells. RNA was analyzed on Northern blots (20μg of RNA per lane). The list of normal cells (lanes 1-4) and tumor cell lines (lanes 5-13) is presented in Table 1 (using identical numbers). **(B)** Western blot analysis of CKIs from cell extracts obtained from the same cell lines used in **A**. 50ug of total cell extract were run on SDS polyacrylamide gels. Proteins were transferred to Immobilon P and blots were incubated with the indicated anti-CKI antiserum and immuno-reactive proteins detected with the ECL reagent (Amersham). Numbers for normal and tumor cell extracts are as described in Table 1.

Figure 5. Expression of CKIs in normal cells synchronized by growth factor deprivation.

Normal 76N cells were arrested in G0 via growth factor deprivation for 72 hours then stimulated to re-enter the cell cycle by adding back of growth factors. **(A)** At indicated times after growth-factor stimulation, whole cell lysates were prepared and analyzed on Western blots. Blots were incubated with indicated anti-CKI antiserum and immuno-reactive proteins visualized with the ECL reagent. Detection time 1-30s with the exception of p16- for 24 hrs- see text). **(B)** DNA synthesis rates as measured by [^{3}H] thymidine incorporation.

Figure 6. <u>Induction of the CKIs p21 and p27 by Lovastatin is inversely correlated with cdk2 kinase activity.</u> MDA-MB-157 tumor cells were cultured in 20uM Lovastatin for 36 hours at which time Lovastatin was replaced by Mevalonate. Samples were collected at indicated times after Lovastatin removal and RNA and proteins extracted.

A: Northern blot analysis: RNA extracted from cells at indicated times after Lovastatin treatment was analyzed by Northern analysis (20 μg/lane) and probed with ^{32}P labeled p21 or P27 cDNA.

B: Western blot analysis: 50ug of Protein extracts from each condition were analyzed by Western analysis with anti-p21 or anti p27 specific antisera and blots developed with the ECL reagent.

C Histone H1 kinase assay: 500ug of extracts were immunoprecipitated with anti-cdk2 antiserum and complexes assayed for the ability to phosphorylate histone H1. The H1 labeling reaction complexes were analyzed by SDS-PAGE and autoradiography.

D: IP/Westerns analysis: 500ug of protein extracts were immunoprecipitated with anti-cdk2 polyclonal antisera and precipitated complexes analyzed by western analysis with anti-p21 or anti-p27 monoclonal antisera.

E: DNA synthesis rates were monitored by [^{3}H] incorporation.

Figure 7. <u>Expression of CKIs in synchronized normal 76N and tumor MDA-MB-157 breast cells.</u> Both cell were synchronized by double thymidine block procedure: 76N cells were incubated in 2mM thymidine for 24 hours, washed and incubated in regular medium for 12

hours and incubated in 2mM thymidine for an additional 24 hours. MDA-MB-157 cells were treated similarly except incubation in thymidine was for 36 hours and thymidine free media for 24 hours. At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to **A**: Western blot, **B**: Histone H1 kinase, and **C**: flow cytometry analyses. 50ug of extracts of cells at indicated times after double thymidine block were subject to Western analysis with antisera directed against the indicated CKI or cyclin A. Note: p21 or p15 +ve C is purified p21 or p15 used as a positive controls. For Histone H1 kinase activity, equal amount of proteins (500 ug) from cell lysates prepared from each cell line at the indicated times were IPed with anti- anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. Panel **B** is the autoradiogram of the histone H1 SDS-PAGE gel **C:** Relative percentage of cells in different phases of the cell cycle for each cell line was calculated from flow cytometric measurements of DNA content. (◆) cells in S phase, (O) cells in G2/M phase, and (❑) cells in G1 phase.

Figure 8. <u>Kinetic analysis of the induction of CKIs in normal and tumor cells by Lovastatin.</u> 76N normal and MDA-MB-157 cells were cultured in 20uM Lovastatin for 36 hours at which time Lovastatin was removed and replaced with fresh media containing 200uM Mevalonate (Mev.).

A: Western Blot: 50ug of extracts of cells treated with Lovastatin alone or Lovastatin followed by Mevalonate at indicated time intervals were analyzed by SDS-PAGE. Proteins were transferred and blots probed with antisera specific for the indicated CKI.

B: Northern Blot: 20 ug of total RNA from Lovastatin treated cells was analyzed by northern analysis and probed with the cDNA corresponding to the indicated CKI.

Figure 9: <u>Growth Curves: MDA-MB-157 Clones</u>

A.The human breast tumor cell line MDA MB 157 was cloned by limiting dilution. Individual cloned populations were selected for growth studies based upon differences in the Western blot

profile for cyclin E, p16, p21 and p27. Cells from each clone were plated at 10 4 cells per well in 6 well tissue culture plates. Time points were counted (by Coulter counter after trypsinization) in triplicate every 48 hours and plotted. B. Doubling times were calculated from logarithmic curves fit to the data.

Figure 1

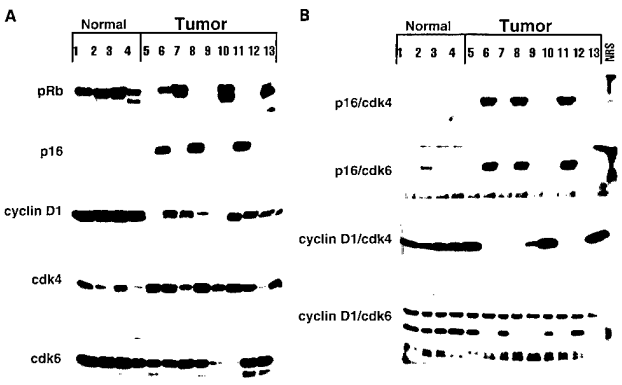

A
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13
pRb
p16
cyclin D1
cdk4
cdk6
B
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13
MRS
p16/cdk4
p16/cdk6
cyclin D1/cdk4
cyclin D1/cdk6

Figure 2

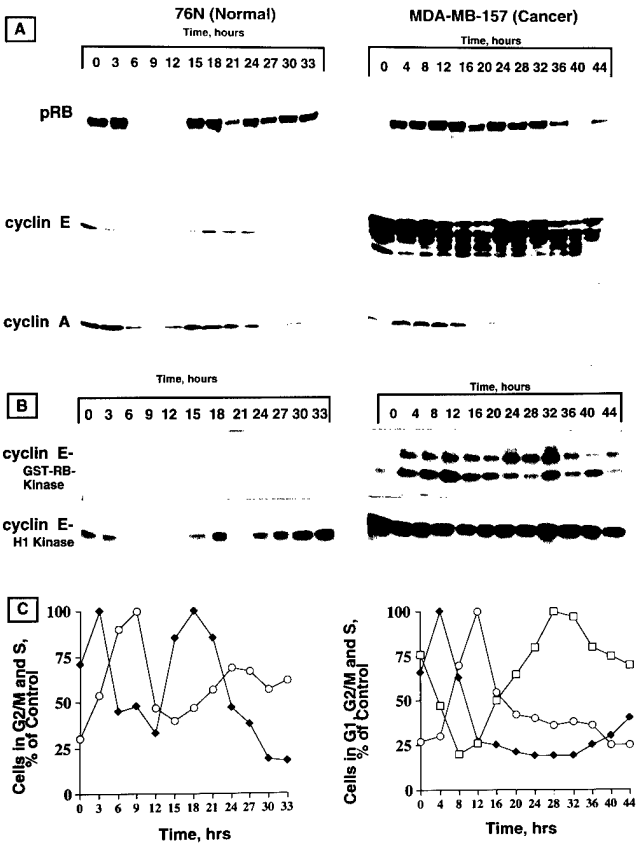

A
76N (Normal)
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
MDA-MB-157 (Cancer)
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
pRB
cyclin E
cyclin A
B
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
cyclin E-GST-RB-Kinase
cyclin E-H1 Kinase
C
Cells in G2/M and S, % of Control
100
75
50
25
0
0 3 6 9 12 15 18 21 24 27 30 33
Time, hrs
Cells in G1, G2/M and S, % of Control
100
75
50
25
0
0 4 8 12 16 20 24 28 32 36 40 44
Time, hrs

Figure 3

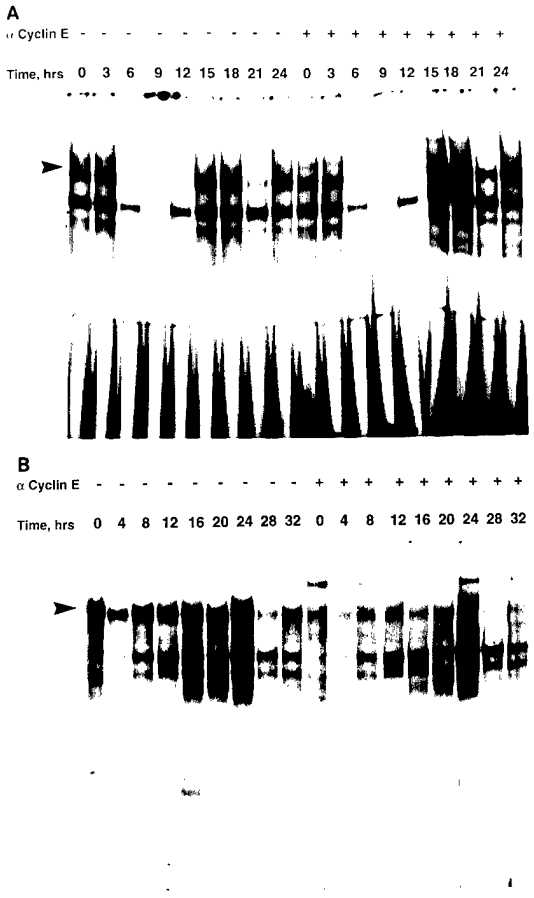

A
α Cyclin E - - - - - - - - - + + + + + + + + +
Time, hrs 0 3 6 9 12 15 18 21 24 0 3 6 9 12 15 18 21 24
B
α Cyclin E - - - - - - - - - + + + + + + + + +
Time, hrs 0 4 8 12 16 20 24 28 32 0 4 8 12 16 20 24 28 32

Figure 4

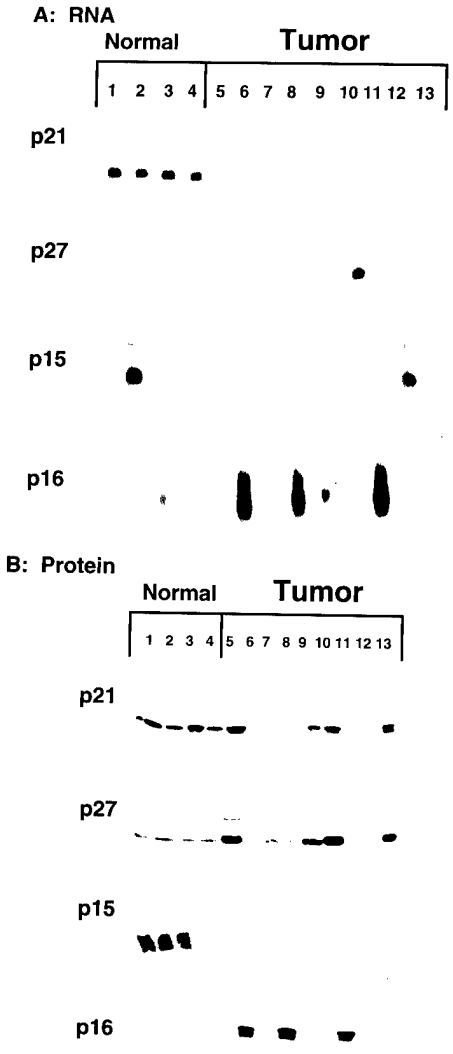

A: RNA
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13
p21
p27
p15
p16
B: Protein
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13
p21
p27
p15
p16

Figure 5

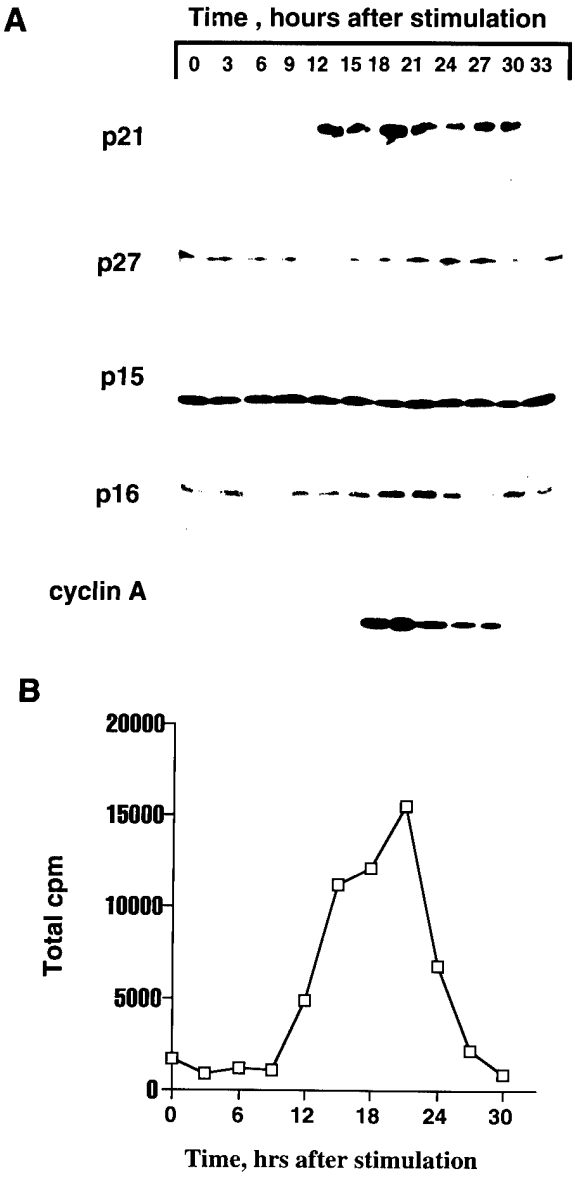

A
Time , hours after stimulation
0 3 6 9 12 15 18 21 24 27 30 33
p21
p27
p15
p16
cyclin A
B
20000
15000
10000
5000
0
Total cpm
0 6 12 18 24 30
Time, hrs after stimulation

Figure 6

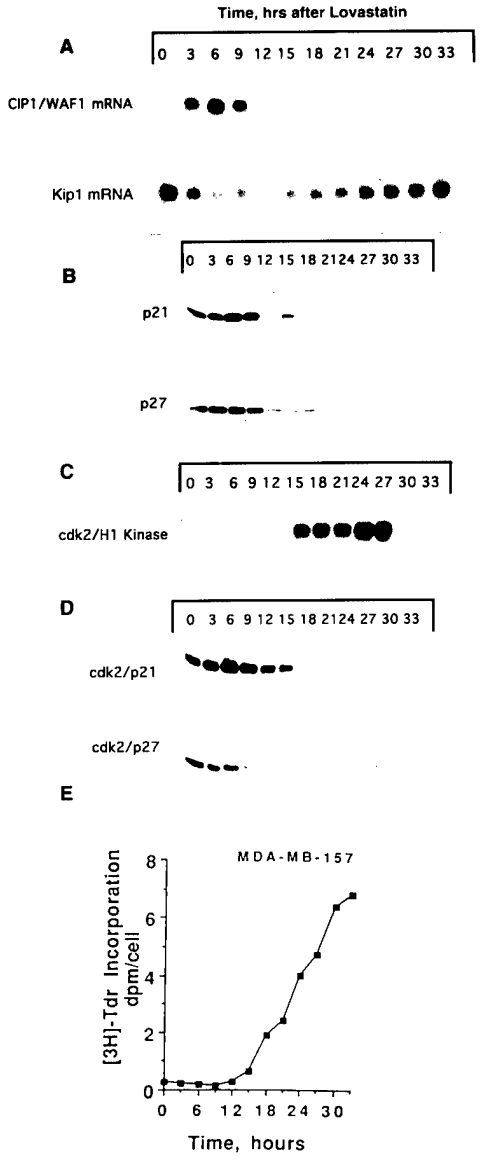
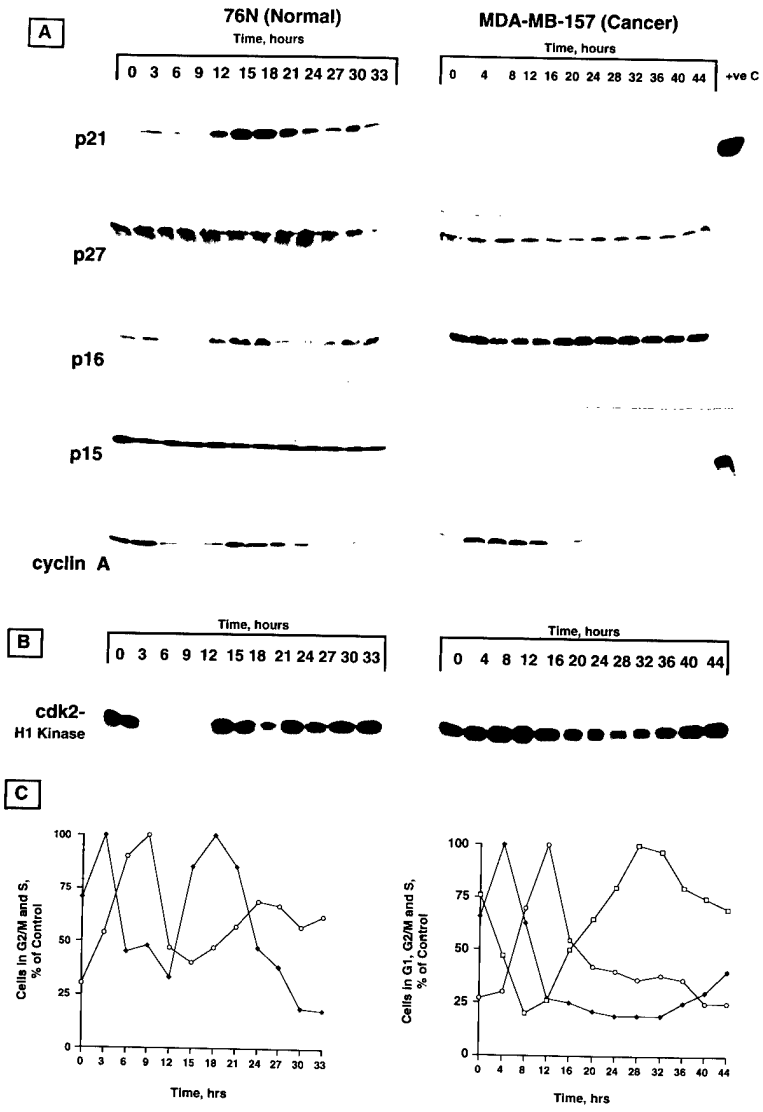
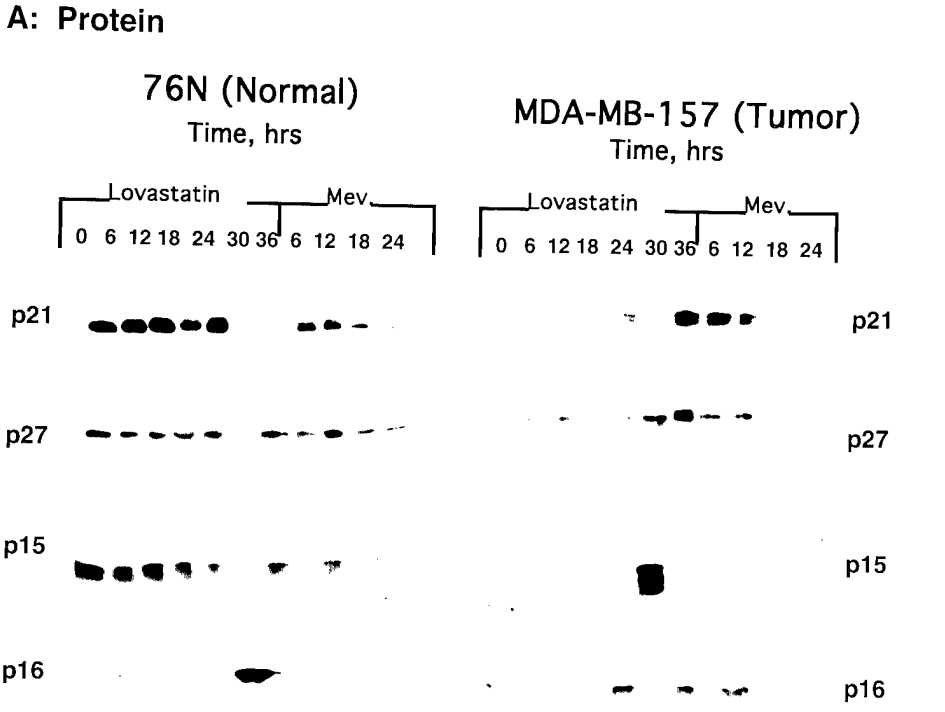

Time, hrs after Lovastatin
A
0 3 6 9 12 15 18 21 24 27 30 33
CIP1/WAF1 mRNA
Kip1 mRNA
B
0 3 6 9 12 15 18 2124 27 30 33
p21
p27
C
0 3 6 9 12 15 18 2124 27 30 33
cdk2/H1 Kinase
D
0 3 6 9 12 15 18 2124 27 30 33
cdk2/p21
cdk2/p27
E
MDA-MB-157
[3H]-Tdr Incorporation dpm/cell
0
2
4
6
8
0 6 12 18 24 30
Time, hours

Figure 7


A
76N (Normal)
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
MDA-MB-157 (Cancer)
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
+ve C
p21
p27
p16
p15
cyclin A
B
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
cdk2-
H1 Kinase
C
Cells in G2/M and S, % of Control
100
75
50
25
0
0 3 6 9 12 15 18 21 24 27 30 33
Time, hrs
Cells in G1, G2/M and S, % of Control
100
75
50
25
0
0 4 8 12 16 20 24 28 32 36 40 44
Time, hrs


A: Protein
76N (Normal)
Time, hrs
Lovastatin
Mev.
0 6 12 18 24 30 36 6 12 18 24
MDA-MB-157 (Tumor)
Time, hrs
Lovastatin
Mev.
0 6 12 18 24 30 36 6 12 18 24
p21
p27
p15
p16
p21
p27
p15
p16

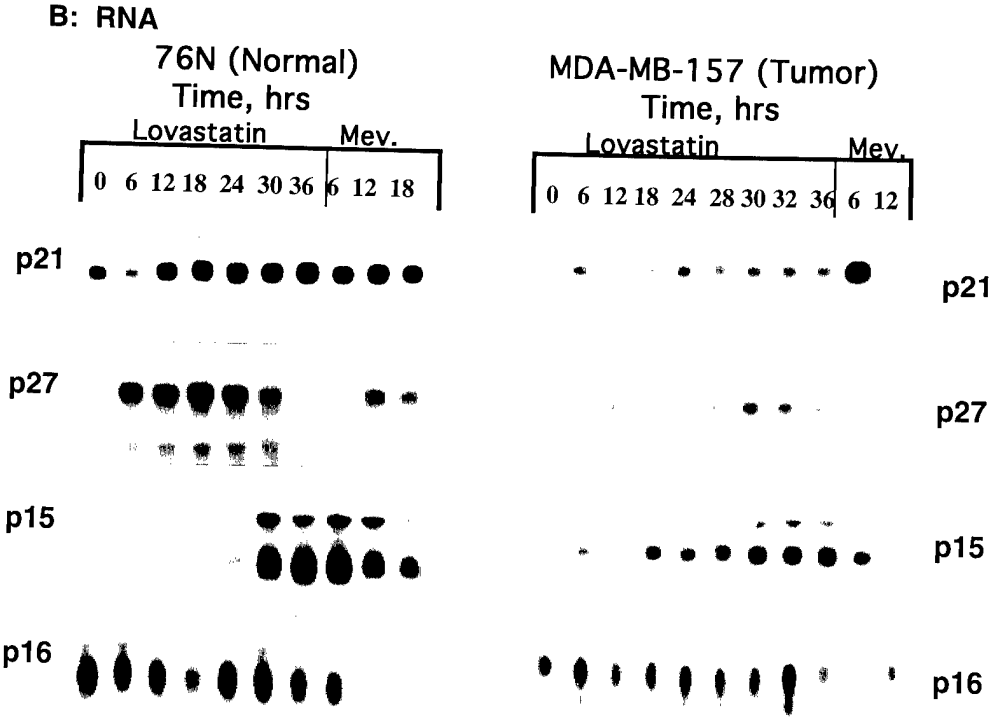
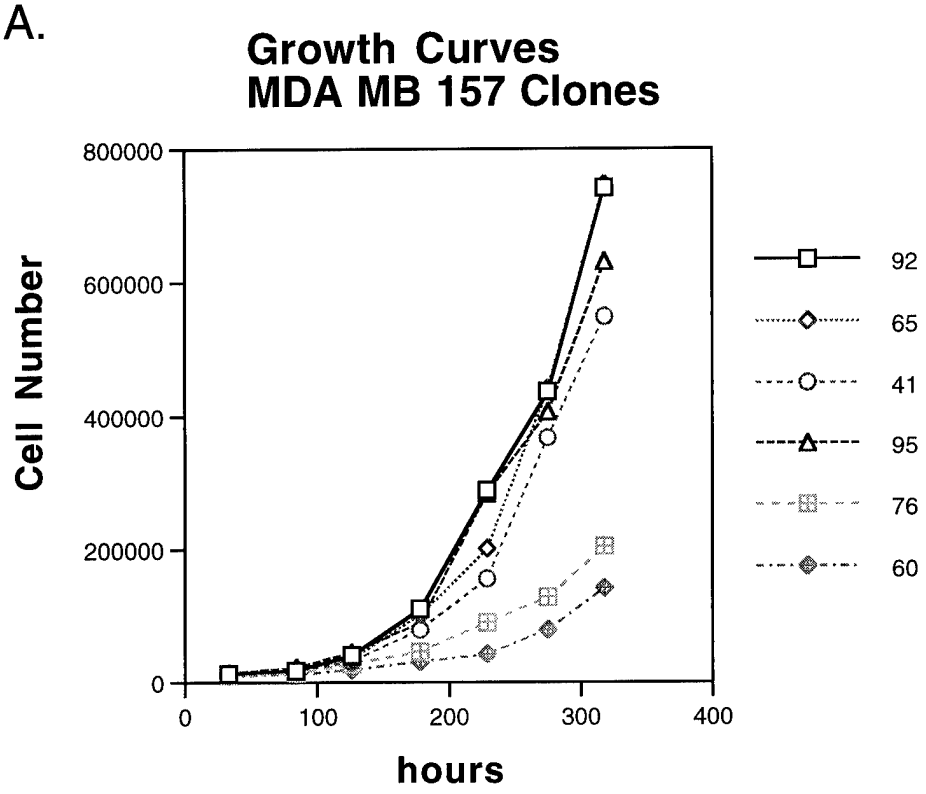

B: RNA
76N (Normal)
Time, hrs
Lovastatin
Mev.
0 6 12 18 24 30 36 6 12 18
MDA-MB-157 (Tumor)
Time, hrs
Lovastatin
Mev.
0 6 12 18 24 28 30 32 36 6 12
p21
p27
p15
p16
p21
p27
p15
p16

Figure 9.

A.



B.

Doubling Times	
Clone 92	45h
Clone 65	46h
Clone 41	48h
Clone 95	48h
Clone 76	66h
Clone 60	74h

Cyclin E — a better prognostic marker for breast cancer than cyclin D?

To the editor — In their recent article, Patricia Steeg and colleagues report that overexpression of cyclin D mRNA occurs in most invasive ductal cancers of the breast, distinguishing invasive breast cancers from nonmalignant lesions[1]. While the findings pertaining to cyclin D mRNA overexpression are timely and important, there was no mention of the prognostic role of another G1 cyclin, namely cyclin E. It was also suggested that cyclin D1, but not E, might function as the growth-limiting restriction-point protein (R protein). We would like to take this opportunity to call attention to the relevant properties of cyclin E, emphasizing its importance in prognosis of early stages of breast cancer and to propose cyclin E as a better candidate for the R protein.

We have documented that altered expression of cyclin E may be associated with breast cancer[2]. Using normal proliferating breast cells versus human tumor breast cells as a model system, we observed a number of alterations in cyclin E expression, including an eightfold amplification of the cyclin E gene in one tumor cell line and aberrant expression in all ten tumor cell lines examined. The deranged production of cyclin E in tumor cells is quantitative and qualitative as cyclin E protein is severely overexpressed in tumor cells and present in lower molecular weight isoforms not observed in normal cells.

We have also extended these observations to the *in vivo* situation by examining the pattern of cyclin E protein expression in tumor and normal adjacent tissues obtained from breast cancer patients[3]. We found that the altered expression of cyclin E protein occurred in most of the breast tumor tissues examined, that the alterations increased with increasing grade and stage of the tumor, and that these alterations were more consistent than c-erb B2 or cyclin D1 overexpression in breast cancer. Furthermore, cyclin E was also altered in other types of solid tumors as well as leukemia. Collectively these observations suggest that the altered expression of cyclin E in the breast tumor samples is not a mere consequence of cell proliferation but represents a significant difference between normal tissue and low- and high-stage tumors and, as such, represents a potential new prognostic marker for breast cancer[3]. Recently we have further extended these studies. We have examined 400 new breast tumor specimens and compared the changes of cyclin E expression with seven other tumor markers, and have shown that cyclin E protein is the most consistent marker for determining the prognosis of early-stage node-negative ductal carcinomas (K.K., manuscript in preparation).

Others have corroborated our findings and demonstrated that immunocytochemical detection of cyclin E detects tumor proliferation and deregulated cyclin expression[4]. The mechanism of the cyclin E alteration is in part a result of its deregulation in breast cancer. Recently, we have documented that while cyclin E protein and its associated kinase activity in normal mammary epithelial cells are cell-cycle regulated, in tumor cells it remains in an active complex throughout the cell cycle[5].

In addition to its role as a prognostic indicator for breast cancer, cyclin E also may function as the R protein. We have proposed three properties to characterize the R protein, as derived from cell biology experiments with mouse 3T3 cells[6].

In cell biological experiments, cycloheximide applied during G1 inhibits total protein synthesis. During a several hour pulse the R protein is lost in normal cells, as a consequence of its instabiltiy. Its resynthesis requires time, and so transit to S phase of these cells is delayed. In contrast, the stable or overproduced R protein in a tumor cell is not degraded and additional delay is not observed[6].

Similarly, pulse-chase experiments were performed in which cyclins E and A and their related kinase activities were measured[7]. By the above criteria, either E or A cyclins could be the R protein. We have recently repeated this experiment with cyclin D and showed that the results were quite different, in that cyclin D protein in both normal and tumor cells disappeared and recovered equally and rapidly with no extra delay of recovery. Therefore, cyclin E (or A) fits our criteria for the R point better than does cyclin D.

QING-PING DOU
Department of Pharmacology
University of Pittsburgh
School of Medicine
W902 Biomedical Science Tower
Pittsburgh, Pennsylvania 15261, USA

ARTHUR B. PARDEE
Dana-Farber Cancer Institute
44 Binney Street
Boston, Massachusetts 02115, USA

KHANDAN KEYOMARSI
Laboratory of Diagnostic Oncology
Division of Molecular Medicine
Wadsworth Center
Empire State Plaza, P.O. Box 509
Albany, New York 12201, USA

Steeg and colleagues reply — We would like to thank Dou, Pardee and Keyomarsi for their informative letter. Our article addressed the mRNA levels of cyclins A and D in premalignant lesions and early carcinomas of the breast. Certainly other cyclins, and other proteins, may contribute to malignant progression. Whether cyclin D or E functions as a "restriction-point protein" is a question that we did not specifically address. We noted the correlation of cyclin D overexpression with any form of carcinoma, and speculated that it may serve proliferative or nonproliferative functions. Given the complexity of cancer development and progression in virtually any cell type studied, it is likely that multiple genetic events are required, and both cyclins D and E may be significant influences.

PATRICIA S. STEEG
Women's Cancers Section
Laboratory of Pathology
Division of Clinical Sciences
National Cancer Institute
Bethesda, Maryland 20892, USA

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Oncogene (1996) 12, 2535–2547

Neu differentiation factor (Heregulin) activates a p53-dependent pathway in cancer cells

Sarah S Bacus[1], Yosef Yarden[4], Moshe Oren[4], Dot M Chin[1], Ljuba Lyass[1], Carolyn R Zelnick[1], Alexander Kazarov[3], Wendy Toyofuku[2], Julie Gray-Bablin[2], Roger R Beerli[5] Nancy E Hynes[5], Mikail Nikiforov[3], Rebecca Haffner[4], Andrei Gudkov[3] and Khandan Keyomarsi[2]

[1]Advanced Cellular Diagnostics, Inc., Elmhurst, IL 60126; [2]Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA; [3]University of Illinois, Chicago, IL 60612, USA; [4]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel; [5]Friedrich Miescher-Institut, Basal, Switzerland

Previously we reported that neu differentiation factor (NDF)/heregulin (HRG) elevates tyrosine phosphorylation of its receptors erbB-3, erbB-4, and erbB-2 (through heterodimer formation). We also showed that both NDF/HRG and antibodies to erbB-2 can arrest growth and induce differentiation in breast cancer cells. In this study, we report on the mechanism of NDF/HRG-induced cellular effects. We show that NDF/HRG and antibodies to erbB-2 receptors up-regulate expression of p53 by stabilizing the protein. This is accompanied by up-regulation of the p53 inducible gene, $p21^{CIP1/WAF1}$, in a variety of cell lines: MCF7 and their derivatives (MCF7/HER2, MN1 and MCF-7-puro), ZR75T and LnCap cells. The induction of p21 is further enhanced when cells are treated with both NDF/HRG and DNA-damaging chemotherapeutic agents (i.e. doxorubicin). The NDF/HRG mediated induction of p21 is dependent on wild-type p53, as it fails to occur in cells expressing dominant negative p53 (MDD2). Furthermore, p21 induction is capable of inactivating cdk2 complexes as measured by Histone H1 phosphorylation assays. Finally, we show that in primary cultures of breast and other cancers, p21 is significantly induced in response to NDF/HRG treatment. Collectively, these observations suggest that the mechanism of breast cancer cell growth inhibition and differentiation via erbB receptors activation is through a p53-mediated pathway.

Keywords: heregulin; Neu; p53; $p21^{WAF1/CIP1}$; cell cycle; erbB-2

Introduction

Cellular growth and differentiation processes involve intercellular communication networks. A primary method through which cells communicate is via growth factors that exert their actions through specific receptors expressed on the surfaces of responsive cells. Ligands binding to surface receptor, such as those which carry an intrinsic tyrosine kinase activity, trigger a cascade of events that eventually lead to cell proliferation and differentiation (Carpenter *et al.*, 1979; Sachs *et al.*, 1987). Receptor tyrosine kinases can be classified into several groups on the basis of sequence similarity and distinct structural features. One of these groups includes the epidermal growth factor receptor family which consists of erbB-1 or (EGFR), (Carpenter *et al.*, 1979) erbB-2 (HER-2/neu) (Semba *et al.*, 1985; Coussens *et al.*, 1985), erbB-3 (HER-3) (Kraus *et al.*, 1989; Carraway *et al.*, 1994) and erbB-4 (HER-4) (Plowman *et al.*, 1993; Tzahar *et al.*, 1994).

ErbB-2 is overexpressed in 20 to 30% of all breast cancers and its overexpression is associated with poor prognosis, suggesting that it can be used as a potential target for anti-tumour agents (Slamon *et al.*, 1987; Tagliabue *et al.*, 1991; Hudziak *et al.*, 1989). Consistent with this hypothesis are studies which report that in erbB-2 overexpressing breast cancer cells, treatment with antibodies specific to erbB-2 in combination with chemotherapeutic agents (i.e. cisplatin) elicits a higher cytotoxic response than treatment with cisplatin alone (Hancock *et al.*, 1991; Arteaga *et al.*, 1994; Pietras *et al.*, 1994). One possible mechanism by which erbB-2 antibodies enhance cytotoxicity to chemotherapeutic agents is through the modulation of tyrosine phosphorylation of the erbB-2 protein (Bacus *et al.*, 1992). Neu differentiation Factor (NDF) is another agent which can also stimulate the tyrosine phosphorylation of erbB-2 through heterodimerization with its receptors erbB-3 or erbB-4 (Tzahar *et al.*, 1994; Plowman *et al.*, 1993; Pinkas-Kramarski *et al.*, 1994). NDF was isolated from the growth medium of Rat1-EJ cells, (Peles *et al.*, 1992) and its human homologue heregulin (HRG) from the growth factor medium of MDA-MB-231 breast cancer cells (Holmes *et al.*, 1992). NDF/HRG can either elicit a growth arrest and differentiation phenotype resulting in morphological changes, induction of lipids, and expression of intracellular adhesion molecule-1, or induce a mitogenic respsonse, depending on the cell line studied (Holmes *et al.*, 1992; Peles *et al.*, 1992; Bacus *et al.*, 1993).

The involvement of growth factors and antibodies to erbB receptors in induction of differentiation, proliferation, as well as in the sensitization of chemotherapeutic agents leading to DNA damage, also predicts the involvement of p53. p53 is one of the most well characterized genes associated with cancer and loss of growth control and is mutated or deleted in a great variety of human tumors (Hollstein *et al.*, 1991). Wild-type p53 acts as a transcription factor for genes regulating growth control and is also involved in the DNA damage induced G1 growth arrest or apoptosis observed in certain cell types (Oren *et al.*, 1992; Symonds *et al.*, 1994; Zhan *et al.*, 1993). The growth arrest function of p53 presumably halts cell cycle progression allowing for DNA repair to occur, thereby

Correspondence: K Keyomarsi
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contributing to the maintenance of genomic integrity (Kastan *et al.*, 1991; Lane *et al.*, 1992). The major p53 inducible transcript in human cells is CIP1/WAF1. This protein was simultaneously characterized in several laboratories as the major inducible gene of the tumor suppressor p53, as a cyclin dependent kinase inhibitor protein, as a protein highly expressed in senescent fibroblasts and as a melanoma-associated gene (El Diery *et al.*, 1993; Gu *et al.*, 1993; Noda *et al.*, 1994; Zhang *et al.*, 1993; Ziang *et al.*, 1993; Sherr *et al.*, 1995; Harper *et al.*, 1993; Xiong *et al.*, 1993). This protein has been shown to be associated with various cyclin-cdk complexes *in vivo* and *in vitro* and can inhibit the kinase activity of ckd2, cdk4 and cdc2 cyclin complexes (Sherr *et al.*, 1995; Luo *et al.*, 1995). p53 induction of p21 in response to DNA damage presumably results in *cdk* inhibition and G1 growth arrest. However, p21 can also be induced in a p53-independent pathway (Halevy *et al.*, 1995; Parker *et al.*, 1995; Zhang *et al.*, 1995).

In this study, we investigated whether p53 and its inducible gene CIP1/WAF1 could contribute to the induction of growth arrest and differentiation by NDF/HRG and to the enhanced cytotoxicity of chemotherapeutic drugs by antibodies to erbB-2. We report here that treatment of cells expressing erbB-2, erbB-3 and erbB-4 with NDF/HRG or antibodies to erbB-2 alone, or in combination with doxorubicin, up-regulates both p53 and p21. Furthermore, NDF/HRG and erbB-2 antibodies have an additive effect when combined with chemotherapeutic agents in up-regulation of p21, resulting in inhibition of cdk2 activity. Finally, we show that primary cultures obtained from breast and endometrial tumors treated with NDF/HRG also up-regulate p21.

Results

Induction of p53 after treatment with NDF/HRG, doxorubicin and N29 in cancer cells

We have previously reported that treatment with NDF/HRG or N29 (an antibody to erbB-2) induces growth inhibition and differentiation of cultured human breast tumor cells (Peles *et al.*, 1992; Bacus *et al.*, 1992). Here we show similar cell cycle responses in MCF7 cells treated with NDF/HRG and the chemotherapeutic drug doxorubicin (Figure 1A). Furthermore, we show that treatment of MCF7 cells with low concentrations of NDF/HRG (5 ng/ml) has a mitogenic effect, while treatments with higher concentrations of NDF/HRG (50 ng/ml) or following doxorubicin treatment (25 ng/ml) cause growth inhibition. Cells accumulate in the G_1 and G_2 phases after 60 h of NDF/HRG or doxorubicin treatment, with a concomitant decrease in the number of cells in the S phase, indicating that proliferation was inhibited (Figure 1B). Furthermore, NDF/HRG at low concentrations (5 ng/ml) induced resistance to growth inhibition by doxorubicin and at higher concentrations (50 ng/ml) increased the growth inhibition caused by doxorubicin, again for the duration of treatments used here (data not shown). Treatment with N29 or doxorubicin also resulted in growth inhibition. Treatment with N29 together with doxorubicin, enhanced the ability of doxorubicin to inhibit growth (data not shown). To determine the possible mechanism of this cellular response, we examined whether these agents could up-regulate the expression of p53 in MCF-7 [which express wild-type p53 as well as erbB-2, erbB-3 and erbB-4 (Siegall *et al.*, 1995)] by both immunohistochemistry (Figure 2, Table 1) and Western blot analysis (Figure 3).

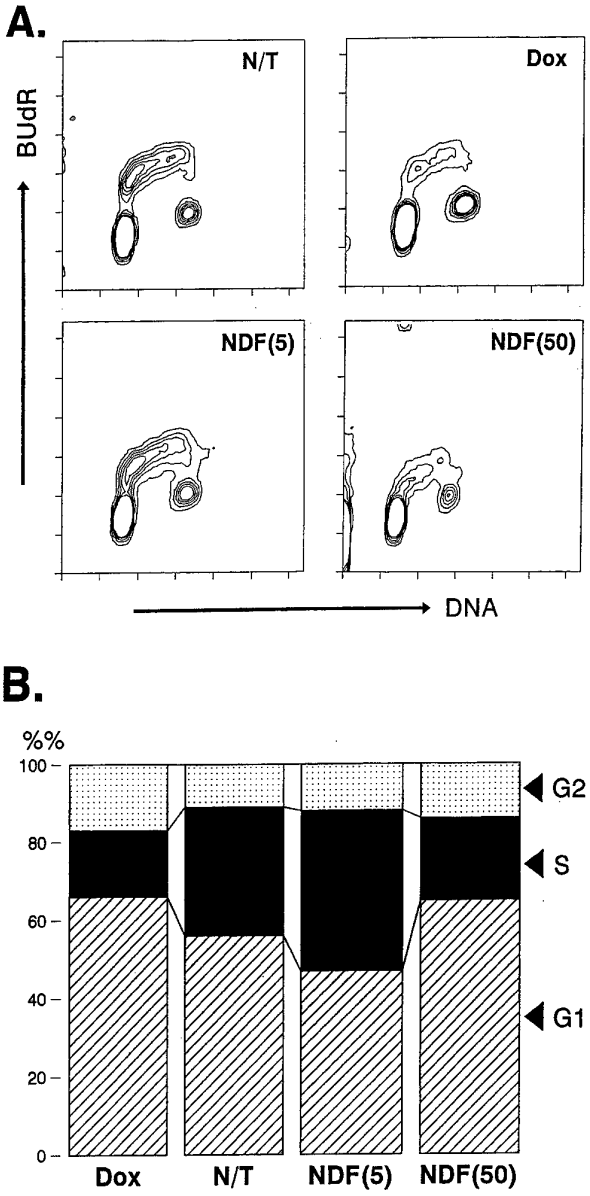

Figure 1 Alterations in the cell cycle of MCF7 cells after treatment with NDF/HRG or doxorubicin. MCF7 cells were treated in the presence or absence of NDF/HRG (5 ng/ml or 50 ng/ml) or doxorubicin (25 ng/ml) for 60 h. Following treatment, BUdR was added for 60 min and cells were then subjected to FACS analysis. (**A**) Two-dimensional FACS analysis of cellular DNA content (propidium iodide staining, X axis, linear scale) and cellular DNA synthesis (BUdR incorporation, Y axis, logarithmic scale). N/T – non-treated cells; NDF(5) – cells treated with 5 ng/ml of NDF/HRG; NDF(50) – treated with 50 ng/ml of NDF/HRG; Dox – cells treated with 25 ng/ml of doxorubicin. (**B**). A histogram depicting the results of the FACS analysis (**A**) of the cell cycle distribution of untreated MCF7 cells or cells treated as described in **A**.

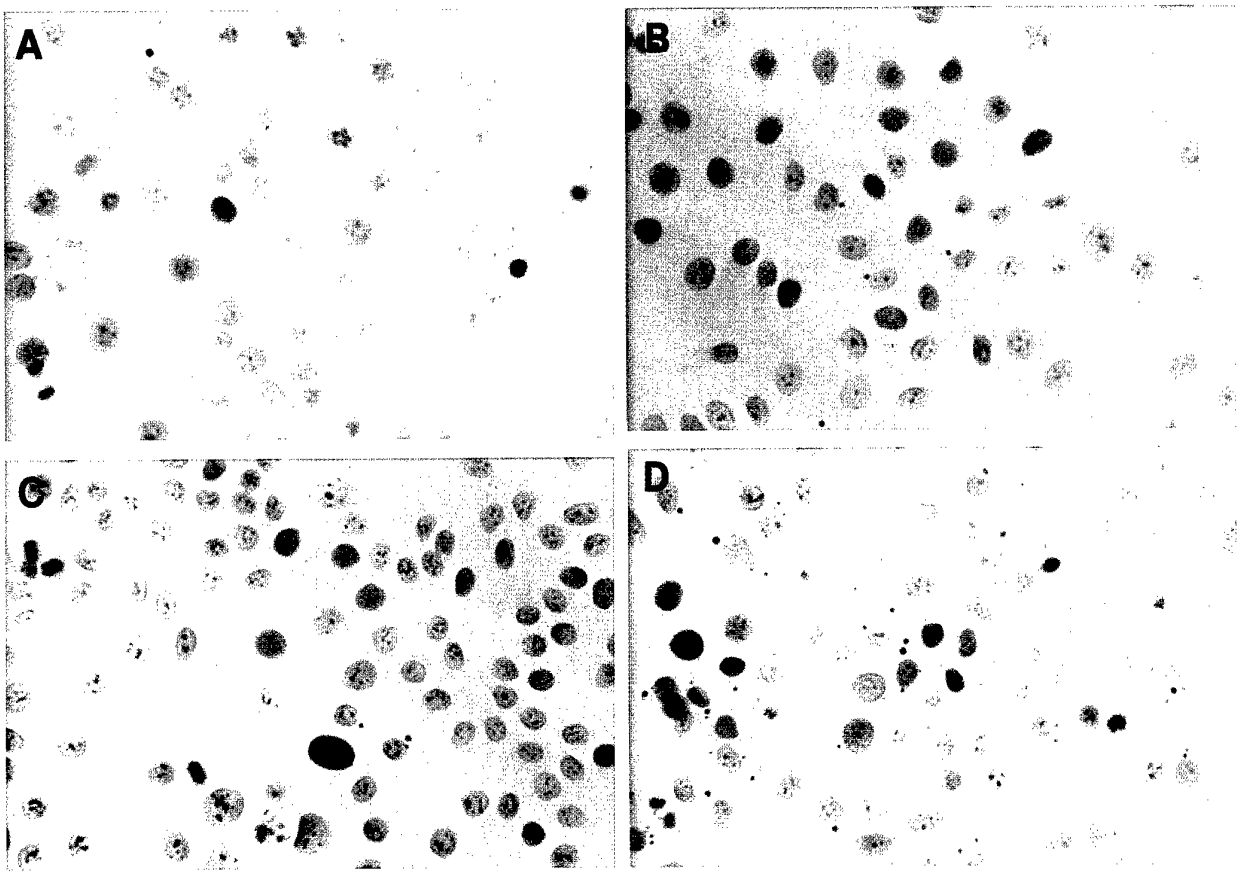

Figure 2 Immunohistochemical staining for p53 in MCF7 cells. Each chamber slide of the Lab-Tek slide was inoculated with 0.5×10^4 cells in 1 ml of medium, NDF/HRG 5 ng/ml or 25 ng/ml of doxorubicin were added after 24 h and the cultures were incubated for an additional 3 days. Cells were fixed and stained by the peroxidase technique using monoclonal antibodies to p53 (Pab 1801, Oncogene Science, Cambridge, MA) and counterstained with methylene blue (**A**) untreated MCF7 cells or treated for 3 days with NDF/HRG (**B**); doxorubicin (**C**); or combination of NDF/HRG and doxorubicin (**D**). Notice the increased percentage of nuclei stained for p53 (brown staining) after the two various treatments. Magnification is ×400

Immunohistochemical localization and quantitative image anlaysis of p53* revealed that in untreated MCF-7 cells less than 10% of the nuclei were stained positively for p53 (Figure 2A). Treatment of cells with NDF/HRG resulted in a two- to threefold increase in the number of cells expressing detectable p53 in a dose dependent manner over a 3-day period (Figure 2B). When cells were treated with doxorubicin alone there was a fivefold increase in cell number expressing p53 (Figure 2C) and when cells were treated simultaneously with both NDF/HRG and doxorubicin, there was an additive increase of p53 expressing cells (Figure 2D). As apparent by the immunohistochemistry, p53 was present predominantly in the nucleus, suggesting that it can perform its function as a transcriptional activator once induced. This effect was also evident when cells were treated with both doxorubicin and antibodies to erbB-2 (N29). Such treatment resulted in a fourfold

*Increase in p53 staining was demonstrated by an increase in the percentage of nuclei staining positively with an antibody to p53, as well as by the strength of the reaction (increase in the optical density staining as revealed by image analysis).

Table 1 Expression of p53 and $p21^{CIP1/WAF1}$ in various cell lines after 3 days of treatment with NDF/HRG

Cell line	*Treatment*	*Cells stained for p53 (%)*	*Cells stained for $p21^{CIP1/WAF1}$ (%)*
MCF-7	ϕ	14.4±5	10.6±4
	NDF/HRG	24.8±7	40.14±8
MN1	ϕ	15.63±2	10±2
	NDF/HRG	25.82±4	37±7
MDD2	ϕ	75.48±6	12±2
	NDF/HRG	77.30±9	13±3
MCF-7/HER2	ϕ	13.7±4	12±3
	NDF/HRG	28.5±3	47±5
LNCAP	ϕ	15±4	11±5
	NDF/HRG	30±8	42±7
ZR75T	ϕ	33.44±4	75.36±5
	NDF/HRG	59.8±10	95.1±4

Percentage of cells stained for p53 (Mab PAb 1801 Oncogene Science, Cambridge, MA) or p21 (Mab EA10, Oncogene Science, Cambridge, MA) in either untreated (ϕ) or cells treated with 5 ng/ml of NDF/HRG for 3 days. Each chamber slide of the Lab-Tek slides was inoculated with 0.5×10^4 cells in 1 ml of medium. NDF/HRG was added 1 day later and the cultures were incubated for an additional 3 days. In each experiment, at least 2000 cells were tested for p53 and p21 expression. The results are the means of four experiments + SEM

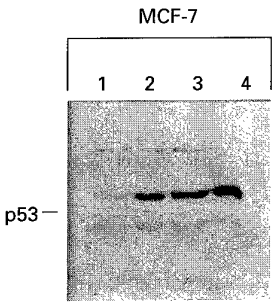

Figure 3 Induction of p53 following treatment with NDF and doxorubicin in MCF7 cells. Cells were treated for 3 days with either no drug (Lane 1), 5 ng/ml β1 NDF (Lane 2), 25 ng/ml doxorubicin (Lane 3), or NDF plus doxorubicin (Lane 4). Following treatments, whole cell lysates were extracted (50 μg of protein extract/lane), run on a 10% acrylamide gel, and blotted as described in Materials and methods. p53 primary antibody was used at a dilution of 1 : 500

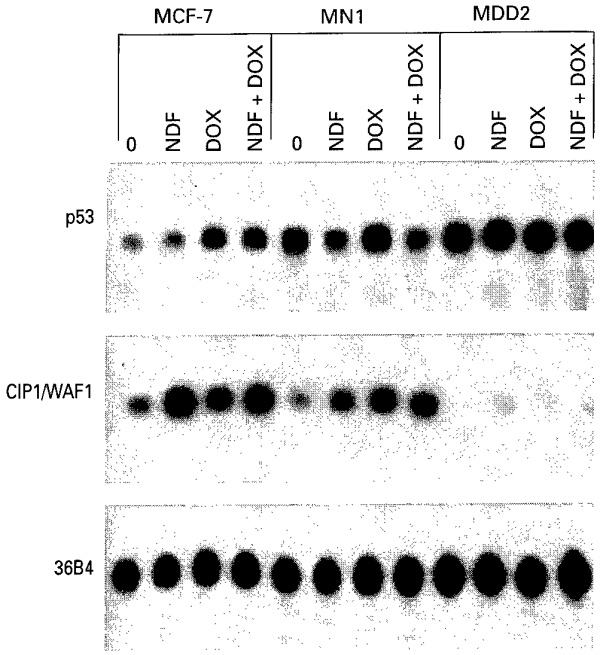

Figure 4 Induction of CIP1/WAF1 mRNA following NDF/HRG treatment. MCF7, MN1 or MDD2 cells were treated with either no drug, 50 ng/ml NEU/HRG, 50 ng/ml doxorubicin, or NDF + doxorubin for 3 days. Following treatments, total RNA was extracted and analyzed on Northern blots (20 μg per lane). Blots were hybridized with the indicated probes or 36B4 used for equal loading (Keyomarsi *et al.*, 1991)

increase of p53 expressing cells, while treatment of cells with N29 alone resulted in only a 1.5-fold increase in cell number expressing p53 (data not shown). A similar increase of p53 expressing cells was observed in other cell lines containing wild-type p53, such as: MCF7/HER-2 cells (MCF7 cells transfected with HER-2/neu), in MN1 cells (generated by stably transfecting MCF7 cells with pSV2neo alone) ZR75T breast cell lines, and in a prostate (LnCap) cancer cell line, all treated with NDF/HRG over a 3-day period (Table 1). The increase in p53 was not observed in MDD2 cells (generated by stably transfecting MCF7 with pSV2neo and pCMVDD expressing a dominant negative p53 miniprotein) which express consistently high levels of p53, presumably due to p53 protein stabilization (Table 1).

Consistent with the immunohistochemical staining, Western blot analysis with p53 antibodies revealed low levels of protein in untreated MCF7 cells (Figure 3, lane 1). Following treatment of cells with NDF, doxorubicin, or NDF plus doxorubicin, p53 levels were increased substantially (Figure 3, lanes 2–4). In MDD2 cells, on the other hand, p53 was present at very high levels with only minor induction upon treatment, confirming the immunohistochemistry results (data not shown). Collectively, these observations suggest that in cells containing wild-type 53, activation of erbB receptors by ligand (NDF/HRG) or antibodies to erbB-2 occurs via pathways used by agents that cause DNA damage, namely p53 pathways.

NEU differentiation factor induces p21 expression in a p53 dependent pathway

We also examined the mode of regulation of p53 expression in epithelial cancer cell lines with different p53 genotypes, cell lines expressing either wild-type p53 (MCF7, MN1), or dominant negative p53 miniprotein (MDD2), by Northern blot analysis (Figure 4). Total RNA was isolated from cultures of MCF-7, MN1, and MDD2 cell lines treated with either 50 ng/ml NDF, 50 ng/ml doxorubicin, or 50 ng/ml each of NDF/HRG + doxorubicin for 3 days and subjected to Northern blot analysis using a probe specific for p53. These analysis revealed that mRNA for p53 was only slightly induced in MCF-7 cells treated with doxorubicin and combination doxorubicin + NDF. Interestingly p53 mRNA was slightly down regulated in MN1 cells treated with NDF or combination, while p53 mRNA remained unchanged in MDD2 cells under all conditions examined. However we already showed that p53 protein was dramatically induced in MCF-7 following NDF/HRG treatment (Figure 3) which is likely due to p53 protein stabilization since no increase in p53 mRNA was detected (Figure 4). To evaluate whether the p53 protein can transcriptionally activate CIP1/WAF1 we reprobed the Northern blots with a CIP1/WAF1 specific probe. These analysis revealed that p21 mRNA levels are clearly induced (fivefold) in MCF-7 cells following NDF treatment and to a moderate degree (threefold) in NDF/HRG treated MN1 cells. MDD2 cells which contain a dominant negative p53 miniprotein express only minimal levels of p21 which were not induced upon treatment with NDF/HRG. Collectively this data depicts that CIP1/WAF1 mRNA is in fact up regulated by NDF/HRG and that this up regulation is p53 dependent as in cells containing a dominant negative p53 miniprotein such induction was not observed.

The increase in CIP1/WAF1 was also seen at the protein level, as shown by immunohistochemically staining for p21 (Tables 1 and 2). Treatment of cell lines with wild-type p53 such as MCF7, MN1, MCF7/Her2, ZR75T and LnCap cells with 5 ng/ml (low dose) of NDF/HRG, increased the percentage of cells staining positively for p21 by 3–5-fold (Table 1). However, p21 protein was not induced in MDD2 cells which express a dominant negative p53 miniprotein

following the same treatment, suggesting that the increase in p21 expressing cells following NDF/HRG treatment is p53 dependent (Table 1). Treatment of MCF-7 cells for three days with doxorubicin (25 ng/ml) also revealed four- to fivefold increase in expression of p21 protein, while treatment with the anti-HER-2 antibody N29 increased p21 expression only by twofold (Table 2). Furthermore, treatment of cells with doxorubicin in combination with NDF/HRG or N29 increased the number of p21-positively staining cells by eight- to tenfold. These results suggest that a combination of erbB-2 activating agents and DNA-damaging agents has an additive effect on induction of p21 in MCF-7 cells through a p53 dependent manner.

ErbB-2 provides high affinity binding sites to cells expressing erbB-3 and erbB-4 (Peles *et al.*, 1993; Tzahar *et al.*, 1994; Karunagaran *et al.*, 1996). The MCF7/5R cells lack surface erbB-2 due to expression of a recombinant erbB-2 antibody in the endoplasmic reticulum of the cells (Beerli *et al.*, 1994; Graus-Porta *et al.*, 1995 and personal communication). In MCF7/puro (MCF7 cells transfected with control vector alone) NDF/HRG significantly increased the number of cells expressing p21 in a dose-dependent manner (Table 3). Treatment of MCF-puro by increasing concentrations of NDF/HRG ranging from 5–100 ng/ml increased the percent cells expressing p21 by 2.6–7.1-fold. However, in MCF7/5R which have reduced affinity to NDF/HRG by limiting expression of erbB-2 receptors to the cytoplasm, treatment with NDF/HRG at concentrations up to 100 ng/ml only minimally increased the already high levels of endogenous p21 (Table 3), suggesting that signal transduction pathways activated by erbB-2 are directly involved in p21 induction. Western blot analyses confirmed the immunohistochemistry results and indicated that MCF7/5R cells treated with NDF/HRG demonstrated only a moderate induction of the initially high basal levels of p21 following treatments. However, in the control cell line, MCF7-puro, NDF/HRG, doxorubicin, and combined treatment substantially up-regulated p21 expression (Figure 5).

Table 2 Expression of p53 and $p21^{CIP1/WAF1}$ in MCF7 cells treated for 3 days with NDF/HRG, N29 and Doxorubicin

Treatment	*Cells stained for p53 (% cells)*	*Cells stained for $p21^{CIP1/WAF1}$ (% cells)*
ϕ	14.43±6	10.60±4
NDF	24.83±7	40.14±8
DOX	23.93±7	50.81±6
DOX+NDF	56.21±10	70.36±5
N29	17.14±9	8.93±4
DOX+N29	50.44±11	78.01±8

Culture conditions, staining and analysis are as described in Table 1. The results are a means of four experiments±SEM

Table 3 Dose dependent induction of $p21^{CIP1/WAF1}$ in MCF7-puro and MCF7-5R cells treated with NDF/HRG for 3 days

Cell line	*Treatment NDF/HRG (ng/ml)*	*Cells stained for $p21^{CIP1/WAF1}$ (%)*
MCF7-Puro	ϕ	9.5
	5	24.60
	50	58.48
	100	67.06
MCF7-5R	ϕ	26.57
	5	30.77
	50	31.92
	100	35.92

Culture conditions, staining and analyses are as described in Table 1. These experiments were repeated twice

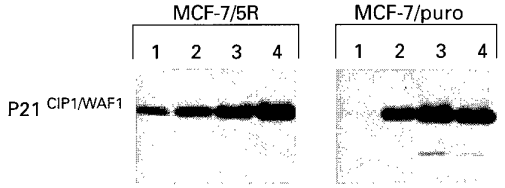

Figure 5 Expression of WAF1/CIP1 in MCF7-puro or MCF7-5R cells. Western blot analysis of p21 in cells treated with no drug, Lane 1; 5 ng/ml NDF, Lane 2; 25 ng/ml doxorubicin, Lane 3, NDF plus doxorubicin, Lane 4. Protein (50 μg per each condition) was applied to each lane of a 13% acrylamide gel and blotted as described in Materials and methods. $p21^{CIP1/WAF1}$ primary antibody was used at a dilution of 1:1000

Biochemical analysis of p21 induction

To investigate the functional significance of p21 induction, we analysed the pattern of expression of p21 in NDF/HRG or doxorubicin-treated cells by Western blot and immune complex kinase assays. These analyses revealed that p21 is indeed induced by NDF/HRG and doxorubicin in a time-dependent fashion and that the combination of NDF/HRG and doxorubicin resulted in an additive induction (Figure 6). Cell extracts from MCF7 cells treated with 5 ng/ml of NDF/HRG, 25 ng/ml of doxorubicin, or combination of the two agents, for 1 or 3 days were subjected to Western blot analysis with an antibody to p21. These analyses reveal that p21 is induced threefold by NDF, fivefold by doxorubicin and 10-fold by NDF plus doxorubicin following 1 day of treatment. When cells were treated for 3 days, p21 remained induced threefold by NDF/HRG, sixfold by doxorubicin and 10-fold by the combination† (Figure 6). To determine whether p27, another universal cdk inhibitor, that is not regulated by p53, (Polyak *et al.*, 1994; Sherr and Roberts, 1995) is also induced by these treatments, we stripped and reprobed the immunoblots with an antibody to p27. These analyses showed that levels of p27 were constant throughout all treatments for the two time intervals examined (Figure 6), suggesting that the induction of p21 by NDF/HRG or doxorubicin is through a p53-dependent pathway.

To examine the functional significance of p21 induction by NDF/HRG and doxorubicin, we measured the phosphorylation of histone H1 in immunoprecipitates prepared from treated MCF7 cell extracts. These analyses, surprisingly, showed the amount of p21 induced by NDF/HRG, following only one day of treatment, was not sufficient to inhibit the cdk2 activity

†The quantitation for all the Western blot analysis were performed by reprobing the Western blots with anti-actin antibody (data shown) and densitometric analysis against actin for each condition was performed.

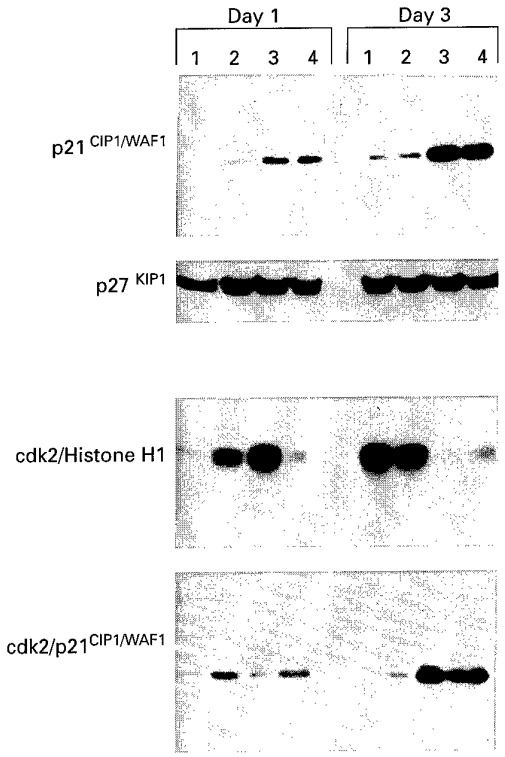

Figure 6 Induction of p21 in MCF7 cells by NDF and doxorubicin results in inhibition of cdk2 activity. Cells were treated for 1 or 3 days with either no drug (Lane 1), 5 ng/ml β1 NDF (Lane 2), 25 ng/ml doxorubicin (Lane 3) or NDF plus doxorubicin (Lane 4). Protein (50 μg) for each time point was applied to each lane of a 13% acrylamide gel and blotted as described. The same blot was reacted with p21 and p27 affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8) and 2% SDS for 30 min at 55°C. for Histone H1 kinase activity, equal amounts of proteins (250 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. Panel marked as cdk2/Histone H1 is the autoradiogram of the histone H1 SDS–PAGE gel. In the panel marked as cdk2/p21 CIP1/WAF1, cell lysates (250 μg) were immunoprecipitated with polyclonal antibody to cdk2 coupled to protein A beads and the immunoprecipitates were washed, boiled for 3 min, separated by SDS-13% PAGE, and blotted to an Immobilon membrane, and hybridized with a monoclonal antibody to p21

in cells treated with NDF/HRG (5 ng/ml) alone. In fact, moderate induction of p21 seemed to have an activating effect on ckd2 function (Figure 6). It was only when cells were treated with doxorubicin alone (for 3 days) or with both NDF/HRG and doxorubicin (for 1 day) that enough p21 was induced to inhibit the cdk2 activity. This observation is more evident in cells treated for 3 days with either doxorubicin (25 ng/ml) or doxorubicin plus NDF/HRG. In these cell extracts, phosphorylation of histone H1 by ckd2 was completely inhibited (Figure 6). There was also an evident increase in both p21 and cdk2/histone kinase activity from day 1 to day 3. This increase was apparently due to cells remaining in the same culture medium for three days and as such were being incubated in condition medium which is capable of increasing cdk2/histone kinase activity. However, even in the presence of the conditioned medium, enough p21 is being induced to fully inhibit the kinase activity.

To determine whether the complexes of cdk2 which inhibited phosphorylation of histone H1, contained p21, we performed sequential immunoprecipitation with cdk2, followed by immunoblotting with p21 (Figure 6). We found only moderate levels of p21 in complexes with cdk2, in extracts from cells treated for one day, while in cells treated for 3 days with doxorubicin or NDF/HRG plus doxorubicin, there were very high levels of p21 in cdk2 complexes. These results suggest that once p21 levels are induced to sufficiently high levels, enough molecules of p21 are able to bind to cdk2 complexes to cause inactivation of the cdk2. These observations are also consistent with the *in vitro* evidence that p21 can act as both an activator and inhibitor of cdk2, depending on how many molecules of p21 are bound in each cdk2-containing complex (Zhang *et al*., 1994; Harper *et al*., 1995).

The induction of p21 is not only time dependent as shown in Figure 6, but it is also dose dependent. Treatment of MCF7 for 3 days with 5 ng/ml of NDF/HRG resulted in only two- to threefold induction of p21, while treatment of MCF7 or MCF7/HER2 cells with 50 ng/ml of NDF/HRG induced a seven- to 10-fold increase in p21, which was comparable to p21 induction by doxorubicin or combined treatment (Figure 7). Treatment with N29 resulted in a modest increase (twofold) in p21. However, N29 had an additive effect on p21 levels when treated in combination with doxorubicin. p27 levels were unchanged under all conditions examined, suggesting that the pathway responsible for induction of p21 is specific to p21, namely the p53-mediated pathway (Figure 7).

To directly determine whether the induction of p21 by NDF/HRG and doxorubicin is via a p53-dependent pathway, we compared the MN1 (transfected with control vector) *vs* MDD2 cells (transfected with dominant-negative p53 sequence) (Figure 8). These cells were treated with NDF/HRG and doxorubicin for 1 and 3 days, and Western blot analyses were

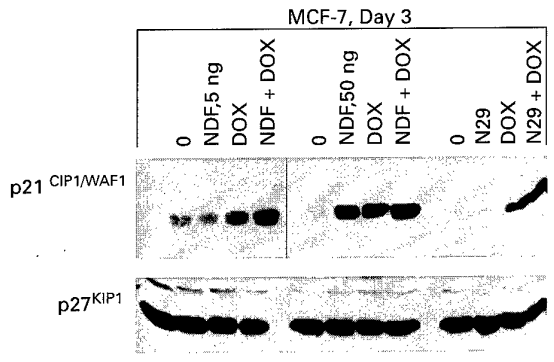

Figure 7 Induction of p21 by NDF is dose dependent. Cells were treated for 3 days with no drug (lanes 1, 5 and 9), 5 ng/ml NDF (lane 2), 50 ng/ml NDF (lane 6), 25 ng/ml doxorubicin (lanes 3, 7 and 11), 5 ng/ml NDF plus 25 ng/ml doxorubicin (lane 4), 50 ng/ml NDF plus 25 ng/ml doxorubicin (lane 8), N29 (lane 10) and N29 plus doxorubicin (lane 12). Protein (50 μg) for each time point was applied to each lane of a 13% acrylamide gel and blotted as described. The same blot was reacted with p21 and p27 affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8) and 2% SDS for 30 min at 55°C

performed with antibodies to both p21 and p27. We found that whereas in MN1 cells, p21 induction was substantial and similar to MCF7 (not transfected), the treatments of MMD2 cells by either NDF/HRG or doxorubicin for 1 day did not induce p21. Only minimal induction of p21 was observed in MDD2 after 3 days of treatment with the combined treatments of NDF/HRG and doxorubicin (Figure 8). Induction of p21 by NDF/HRG depends on the expression of erb-3 and erb-4, providing binding sites for NDF/HRG. In MN1 and MDD2 the expression of the erbB receptors were not affected by transfection of the cells with dominant negative p53 or a control vector. Thus the lack of induction of p21 in MDD2 cells is due to the expression of the dominant negative p53. P27 levels were unchanged under all conditions examined in both cell lines.

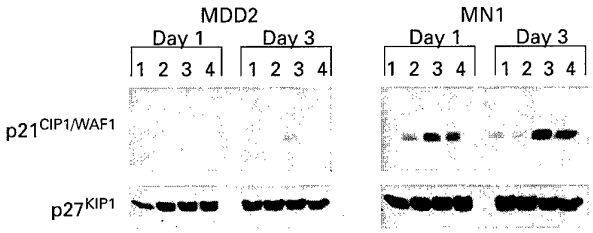

Figure 8 Induction of p21 by NDF and doxorubicin is p53-dependent. MDD2 (dominant negative p53) or MN1 cells (mock transfected MCF7 cells, wild-type p53) were treated for 1 or 3 days with either no drug (Lane 1), 5 ng/ml β1 NDF (Lane 2), 25 ng/ml doxorubicin (Lane 3), or NDF plus doxorubicin (Lane 4). Protein (50 μg) for each time point was applied to each lane of a 13% acrylamide gel and blotted as described. The same blot was reacted with p21 and p27 affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8) and 2% SDS for 30 min at 55°C

Induction of p21 in primary cultures

In order to directly correlate the *in vitro* (cell lines) phenomenon of p21 induction by NDF/HRG through a p53-dependent pathway to the *in vivo* condition, primary cultures of breast and endometrial cancers expressing wild-type p53 were treated with NDF/HRG. These analyses revealed that p21 was in fact induced in these primary cultures by NDF/HRG (Figure 9 and Table 4). Minimal staining for p21 was expressed in untreated primary cells derived from freshly isolated breast tumors (Figure 9, Table 4). Treatment of primary breast cancer cells, expressing normal levels of erbB-2, erbB-3 and erbB-4 with doxorubicin increased the number of cells expressing p21 by sixfold, treatment with NDF/HRG increased p21 expressing cells by fourfold; while treatment with both agents increased p21 expressing cells over ninefold (Table 4). In one primary tumor that overexpressed erbB-2 and erbB-3 and demonstrated normal expression of erbB-4, doxorubicin (25 ng/ml) or NDF/HRG (5 ng/ml) treatments resulted in a four- to sixfold increase of p21-expressing cells, respectively (Figure 9C–E) and the combined treatment of both agents, as well as the combined treatment of N29 and doxorubicin, resulted in up to a ninefold increase of p21-expressing cells (Figure 9F). Treatment of primary cells obtained from breast cancer cells expressing mutated p53 with NDF/HRG or doxorubicin failed to up-regulate p21 even when the tumor expressed high levels of erbB-2 and erbB-4 (Table 4). The up-regulation of p21 by NDF/HRG was also observed in other primary cancer cells (endometrial cancer) (Table 4). Collectively, these observations suggest that the induction of p21 by NDF/HRG, doxorubicin, or the combination of both agents is via a p53-dependent pathway and is evident both in established cell lines as well as cells derived from primary tumors.

Table 4 Induction of $p21^{CIP1/WAF1}$ in primary cultures. ErbB receptor levels (Arbituary Units)

					% Cells stained for $p21^{CIP1/WAF1}$ Treatment			
Tissue	*HER2*	*HER3*	*HER4*	*Positive p53 Staining*	*NT*	*NDF*	*DOX*	*NDF/DOX*
NDF/DOX								
Breast; Infiltrating ductal carcinoma, 95–222	0.5	0.75	0.43	–	10%	40%	68%	98%
Breast; Ductal carcinoma *In situ*, 95-475	3.6	5	0.92	–	8%	60%	40%	96%
Breast; Infiltrating ductal carcinoma, 95-583	3.0	0.4	1.8	+	15%	18%	14%	15%
Endometrium; Poorly differentiated adenocarcinoma of the endometrium	–	2	2.7	–	25%	40%	55%	85%
*Breast; Infiltrating ductal carcinoma, 95-474	1.3	1.6	1.3	–	–	+*	+*	+*
Breast; Infiltrating ductal carcinoma, 95-434	0.65	–	1.6	+	25%	24%	26%	28%
Breast; Infiltrating and intra-ductal carcinoma, 95-725	0.83	0.83	0.94	–	6%	42%	70%	90%

+*$p21^{CIP1/WAF1}$ upregulated; too few cells were present to give a percentage. **Receptor levels were terminated by image analysis following detection with specific MAb- 10 000 optical density units corresponded to 1 unit of receptor amount. ***Primary cultures were derived from fresh tumors as described in Materials and methods. Cells were treated for 16 h, fixed and stained for $p21^{CIP1/WAF1}$ as decribed in Table 1. Abbreviations of treatment: NT- Not Treated; NDF- Treated with 5 ng/ml of NDF; DOX- Treated with 25 ng/ml of Doxorubicin; NDF/DOX- Treated with 5 ng/ml of NDF + 25 ng/ml of Doxorubicin

Discussion

NDF/HRG, a ligand to erbB-3 and erbB-4, stimulates tyrosine phosphorylation of its receptors, inhibits proliferation, induces differentiation, or acts as a mitogen in some breast and neuronal cells. Furthermore, EGF and antibodies directed to erbB-2, or EGFR, have been shown to enhance the sensitivity of breast and ovarian cancer cells to cisplatin, doxorubicin, mitomycin C or radiation therapy (Hancock *et al*., 1991; Arteaga *et al*., 1994; Pietras *et al*., 1994; Amagase *et al*., 1990; Kwok *et al*., 1989, 1991; Wu *et al*., 1995). These observations led to the hypothesis that NDF/HRG induction of differentiation or the sensitization to chemotherapeutic drugs by antibodies or ligands to erbB receptors could involve the p53 pathway, as all the treatments listed above could lead to DNA damage. In the present study, we tested this hypothesis and show that, indeed, treatment of cells expressing wild-type p53 and erbB-2, erbB-3 or erbB-4 with NDF/HRG elevated long-term expression of p53 and p21, while similar treatment failed to up-regulate their expression in cells devoid of wild-type p53. In addition, p21 was also up-regulated by a specific antibody directed to erbB-2 (N29), with further induction observed by DNA-damaging agents. The

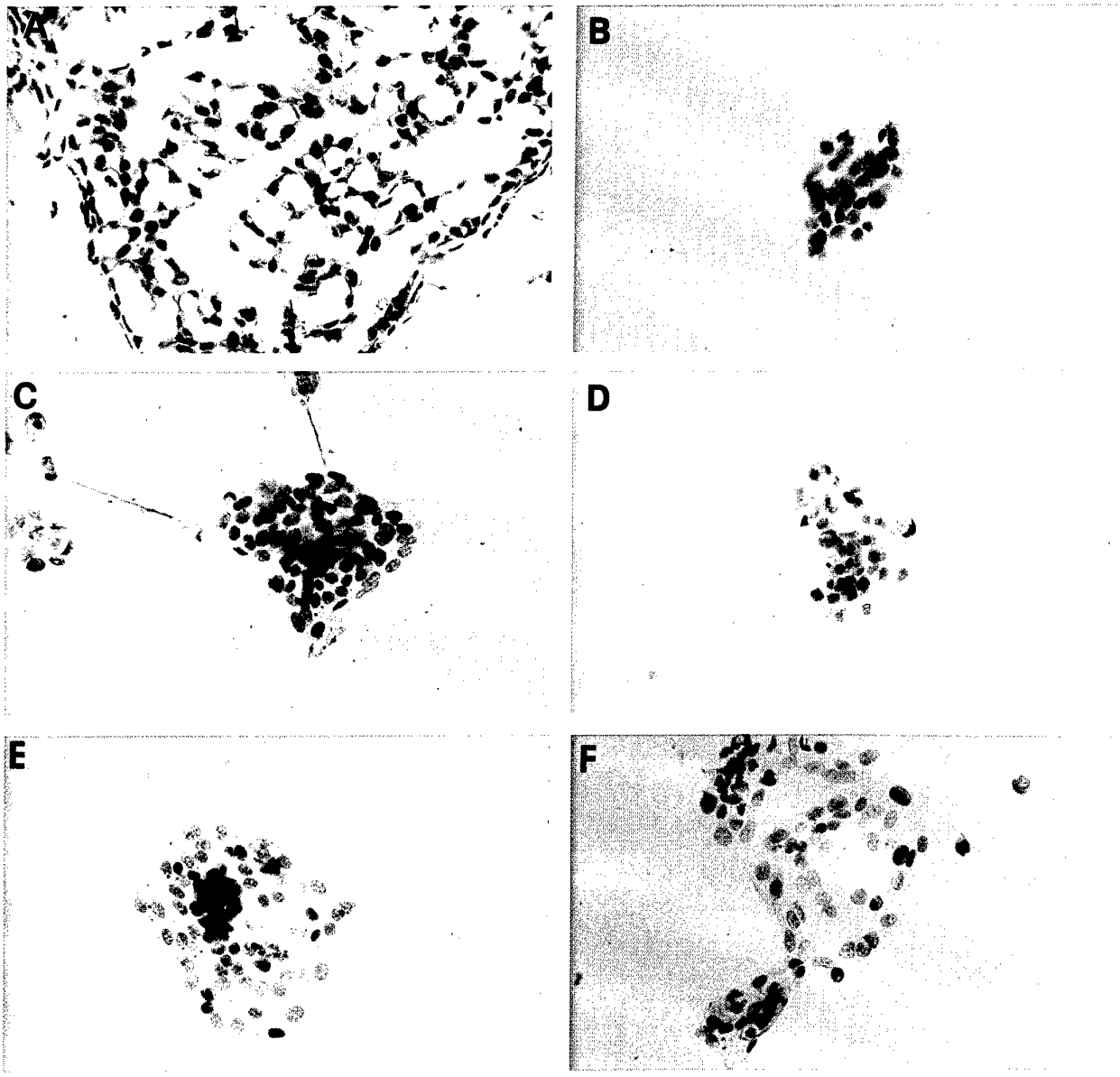

Figure 9 Induction of $p21^{WAF1/CIP1}$ in primary breast cancer cultures. (**A**); erbB-2 staining using a antibody to erbB-2 (N24, Bacus *et al*., 1992) by the alkaline phosphatase technique (red stain) and counter stained for DNA for the feulgen technique (blue stain) of a tissue section derived from a portion of the erbB-2 overexpressing breast cancer tumor used for primary culture. (**B**–**F**); primary cell culture. Fresh specimen was dissociated and its cells were cultured *in vitro*, treated and stained for p21 by the peroxidase technique using monoclonal antibodies to p21 (EA70, Oncogene Science, Cambridge, MA) and counterstained with Methylene Blue. (**B**); control untreated cells, (**C**); cells treated with NDF/HRG (5 ng/ml), (**D**); cells treated with doxorubicin (25 ng/ml), (**E**); cells treated with NDF/HRG and doxorubicin, (**F**); cells treated with N29 (10 μg/ml) and doxorubicin. Magnification is $\times 200$

induction of p21 by NDF/HRG was also evident in primary cultures, as treatment of primary cultures of breast (and endometrial) tumors, expressing wild-type p53, led to a significant up-regulation of p21. Although treatment of MCF7 cells by N29 alone resulted in a slight increase in p21, treatment with N29 and doxorubicin resulted in an enhanced induction of p21, compared to treatment with doxorubicin alone. These results could be due to the inhibition of DNA repair by N29 after doxorubicin mediated DNA damage, resulting in p21 induction. Consistent with this hypothesis is a report describing the ability of 4D5, another antibody to erbB-2, to inhibit DNA repair following cisplatin treatment (Pietras *et al.*, 1994). Although p21 has been reported to be also induced through a p53-independent pathway (Halevy *et al.*, 1995; Parker *et al.*, 1995), NDF/HRG failed to induce p21 in cells expressing dominant negative p53 (MDD2) or in primary cultures derived from patient's tumors expressing mutant p53. In addition, NDF/HRG and N29 have been reported to induce differentiation in some breast cancer cell lines (Bacus *et al.*, 1992, 1993). Consistent with these findings the up-regulation of p53 in HL-60 cell lines undergoing differentiation has been recently reported (Ronen *et al.*, 1996). Thus, induction of differentiation by NDF/HRG could also be associated with induction of p53 and p21.

Even though NDF/HRG has been shown to bind directly to erbB-3 and erbB-4, it also has been shown to phosphorylate and down-regulated erbB-2. This process can be mediated through erbB-2 receptor dimerization with either erbB-3 or erbB-4, resulting in increase in tyrosine phosphorylation of the erbB receptors and in the rate of receptor endocytosis. Subsequently, the overall enzymatic activity of the constitutively active kinase will decrease and result in growth arrest or differentiation (Peles *et al.*, 1991, 1992), which may be accompanied by long-term induction of p53 and p21 as we report here. NDF/HRG induced a moderate induction of p21 mRNA and no induction of p53 mRNA. However, consistent with our results, previous studies have reported that significant levels of wild-type p53 and p21 proteins are induced after MCF7 cells are exposed to DNA-damaging agents, which resulted in only small increases in p21 mRNA. The discrepancy in the amount of mRNA levels *vs* p53 and p21 protein levels is thought to be due to increased translation of p21 mRNA following DNA damage (Kuerbitz *et al.*, 1995; Zhan *et al.*, 1993; Sheikh *et al.*, 1995; Macleod *et al.*, 1995; Gudas *et al.*, 1995). These studies are examples of how low induction of p21 or p53 mRNA may contribute to high levels of protein in a p53-dependent fashion. As such, the moderate induction of p21 mRNA followed by a significant induction of p21 protein as a result of treatment of cells with NDF/HRG is probably due to post-transcriptional stabilization of p21 protein in a p53-dependent fashion.

Treatment of MCF7 cells with low concentrations of NDF/HRG (5 ng/ml) acted as mitogen whereas high concentrations of NDF/HRG (50 ng/ml) acted as a growth inhibitor. More over, in MCF7 cells treated with low concentrations of NDF/HRG together with 25 ng/ml of doxorubicin, NDF/HRG acted as a survival factor and inhibited doxorubicin action. A recent study of PC12 cells (Xia *et al.*, 1996) has shown an opposing effect of mitogen-activated protein kinase (MAP kinase) (which is increased by treatment with NDF/HRG, Karunagran *et al.*, 1996), and the stress-activated JNK pathways. Therefore, the dynamic balance between NDF/HRG-activated MAP kinase and JNK pathways may be important in determining whether NDF/HRG treatment, alone or together with doxorubicin, will cause cells to survive or to undergo apoptosis. This also may explain the fact that patients overexpressing erbB-2 (and express consistently high levels of MAP kinase) are resistant to treatments with low levels of doxorubicin but do respond to high levels of the drug (Muss *et al.*, 1994).

Several lines of evidence suggest crosstalk between the p53 pathway and the epidermal growth factor family of proteins: (i) treatment of MCF7 or MCF7/HER-2 cells with high levels of NDF/HRG (50 ng/ml) induced WAF1/CIP1 seven- to 10-fold *vs* two- to threefold induction by low levels of NDF/HRG (5 ng/ml) (Figures 6 and 7 of this study). (ii) NDF/HRG failed to substantially up-regulate p21 in cells which do not express erbB-2 on the plasma membrane (MCF7-5R) (Table 3, Figure 5 of this study). NDF/HRG only minimally up-regulated p21 in MCF7/5R as other receptors to NDF/HRG, such as HER3 and HER4, were still present in the membrane. Since HER2, by heterodimerization with HER3 or HER4, provides high affinity binding site to NDF/HRG (Karunagaran *et al.*, 1996) there was a lower induction of p21 by NDF/HRG in MCF7/5R when compared to MCF7/puro cell lines (which express erbB-2 on their plasma membrane). Table 3 shows that treatment of MCF7/puro cells with increasing concentrations of NDF/HRG (10–100 ng/ml) results in up to seven-fold induction of p21. The same treatment of MCF7/5R cells results in only 1.4-fold induction. Similarly, Western blot analysis, which compares the effect of NDF/HRG and doxorubicin on the induction of p21, shows that while in MCF-puro cells p21 is induced substantially (10-fold) by NDF/HRG, doxorubicin, or NDF/HRG + doxorubicin, MCF7/5R shows only a modest (2–3-fold) induction by these treatments. (iii) Induction of p21 by NDF/HRG in primary cultures derived from erbB-2 and erbB-3 overexpressing breast cancer was much higher than in those cultures not overexpressing these two receptors and the induction was dependent on wild-type p53, as NDF/HRG failed to induce p21 in primary cultures derived from cancers with mutated p53 (Figure 9, Table 4 of this study).

The mechanism of induction of p53 and p21 by NDF/HRG may involve the activation of the two major cellular signaling pathways – the mitogen activated protein kinase (MAP kinase) and the Jun kinase (JNK). Treatment of MCF7 cells with NDF/HRG activated MAP-kinase (ERK) and the Jun kinase (JNK), and ligand activation was enhanced and prolonged in cells overexpressing erbB-2 (Karunagaran *et al.*, 1996). In addition, regulation of p21 expression through MAP kinase signaling pathways has been reported recently (Liu *et al.*, 1996). Thus, changes in activation of MAP kinase may be involved in the NDF/HRG dependent up-regulation of p21. This also may explain the fact that changes in MAP kinase due to changes in expression of erbB-2 on the plasma membrane in MCF7-5R cells may have resulted in higer basal levels of p21. In MCF7 cells, erbB-2 acts

as a high affinity receptor sub-unit that potentiates the signaling of NDF/HRG. The amount of induction of p21 through the MAP kinase pathway by NDF/HRG may be dependent on high-affinity binding of NDF/HRG by erbB-2. ErbB-2 overexpression has been reported to enhance binding affinities to both EGF and NDF/HRG, through deceleration of ligand dissocation rates (Karunagaran *et al.*, 1996), and removal of erbB-2 from the cell surface almost completely abolished ligand binding by accelerating dissociation of both growth factors. The kinectic effects resulted in enhancement and prolongation of the stimulation of MAP-kinase and c-Jun kinase (SAPK) or NDF/HRG.

Our results lead us to propose that following NDF/HRG treatment, cells can diffferentiate or proliferate through a p53-mediated pathway. Transient activation by such treatment results in a low level of p21 induction, which is not effective in the inhibition of cyclin kinases, in particular, cdk2. However, sustained activated results in induction of higher levels of p21 protein, presumably resulting in several p21 molecules binding to cyclin/cdk2 complexes, leading to inactivating of cdk2. Differences in ligand concentrations or the numbers of erbB receptor molecules could lead to sustained *vs* transient activation. Prolonged activation of MAP-kinase has been shown to induce differentiation, and its transient activation leads to induction of cellular growth (Traverse *et al.*, 1994; Dikic *et al.*, 1994; Marshall, 1995). Future experiments examining regulation of $p21^{WAF/CIP1}$ expression through the MAP kinase pathways by NDF/HRG or by antibodies to erbB receptors could clarify the role of MAP-kinase in this p53 pathway. Finally, our results may indicate that assay of p53 activity as manifested by the up-regulation of p21 in a patient's tissue, after chemotherapy or in combination with antibody treatment, may identify a patient's response to therapy. Hence, NDF/HRG, antibodies and growth factors directed to the erbB family members that up-regulate p53 pathways, could be identified as potential agents to be used in combination with chemotherapeutic agents, for treatment of breast cancers.

Materials and methods

Chemicals and reagents

Doxorubicin and cisplatin (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide and stored at −70°C. Neu differentiation factor (NDF/HRG), and N29 (antibodies directed to the extracellular domain of erbB-2) (Bacus *et al.*, 1992) were obtained from the Weizmann Institute (Rehovot, Israel). NDF/HRG is a 44 kDa glycoprotein which stimulates tyrosine phosphorylation of erbB2 (Peles *et al.*, 1993). The primary structure of NDF/HRG indicates that these molecules comprise a new family of polypeptide factors. These mosaic proteins are encoded by a single gene (on chromosome 8). The basic structure of NDF/HRG includes N-terminal region, an immunoglobulin (Ig) motif, a spacer domain, an EGF like domain, a transmembrane domain, and a cytoplasmic tail (Peles *et al.*, 1993). However, many variations of this structure exist. The two major subtypes of NDF/HRG denoted α and β are classified according to the terminal 18–21 amino acids of the EGF like domain. The subtype 1 through 5 are classified according to the juxtamembrane sequence distal to the EGF like motif. Ligand binding analysis have shown that β1 NDF/HRG isoform binding affinity to erbB-3 and erbB-4 is 5–8-fold higher than any of the α NDF/HRG isoforms (Wen *et al.*, 1994; Karunagaran *et al.*, 1996). Therefore, for all our experiments we have used β1 NDF/HRG for induction of p21. However, data from our laboratory and others (Wen *et al.*, 1994) have shown that both isoforms have a similar biological activity. Treatment with doxorubicin, NDF/HRG or N29 was initiated 24 h after cell plating and was continued for 1–3 days. The final concentration of dimethyl sulfoxide (0.1%) in growth media did not affect cell growth or induction of p53 or WAF1/CIP1.

Cells and culture conditions

LnCAP and ZR75T were purchased from the American Type Culture Collection (Rockville, MD). MCF7 cells were obtained from the Michigan Cancer Foundation (Detroit, MI). MCF7/HER-2 are MCF7 cells with five- to eightfold elevated expression of erbB-2 through erbB-2 gene transfer (Peles *et al.*, 1993). Another cellular system comprised of two derivatives of the MCF7 mammary cancer cell line that has moderate erbB-2 expression: MCF7-puro; a vector alone control cell line, and MCF7-5R cells that display no erbB-2 at the cell surface, as a result of ectopic expression in the endoplasmic reticulum of an engineered single chain monoclonal antibody to erbB-2. Both cell lines were obtained from the Friedrich Miescher Institut (Basal, Switzerland) (Beerli *et al.*, 1994). MN1 and MDD2 derivatives of MCF7 cells were generated in a similar manner as previously described (Shaulian *et al.*, 1992). Briefly, MCF7 cells were maintained in DMEM + 10% fetal calf serum. To generate MDD2 cells, MCF7 cells were cotransfected (by the calcium phosphate coprecipitation) with 15 μg of a plasmid containing the pCMVDD p53 miniprotein and 2 μg of a plasmid containing the neo resistance gene. pCMVDD p53 expresses a short C-terminal segment of p53 and forms stable oligomers with coexpressed full-length p53. The functional consequence of the formation of such mixed oligomers is the abrogation of sequence-specific DNA binding by the wt p53 (Shaulin *et al.*, 1992). Plates were split 24 h after transfection in a 1 : 10 ratio and selected for a period of 3 weeks in medium containing 450 ug/ml G418. Selected clones were expanded and screened for expression of DD-p53 miniprotein. MN1 cells were generated by transfection of the neo resistance plasmid alone, followed by G418 selection. MN1, MDD2, MCF7 and their derivatives (except MCF7/5R) cells express all four members of the erbB family, as demonstrated by immunohistochemical staining using specific antibodies to the various receptors (data not shown). Staining for p53 demonstrated that MCF7 cells, as well as their derivatives (except MDD2), retained wild-type p53. All of the cells were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified incubator with 8% CO_2 in air at 37°C. For the various experiments, exponentially growing cells were plated into Lab-Tek eight-well chamber slides (Nunc, Naperville, IL) or into 100 mm petri dishes at 1×10^4 cells/ml. Cell numbers were determined by hemocytometer chamber counting, and viability was monitored by exclusion of trypan blue dye.

Primary cultures of breast cancers

Fresh tumors were rinsed and finely minced in M15 medium after necrotic areas and blood clots were removed. The cell-containing M15 medium was filtered through a Falcon 80-μm nylon mesh cell strainer and washed three times. The cells that passed through the filter were washed and

centrifuged three times at 1000 r.p.m. for 5 min. Trypan blue exclusion was used to determine cell number and viability. Cells were then plated in M41 medium on collagen-coated chamber slides (Nunc, Naperville, IL) as previously described (Bacus *et al.*, 1993). NDF/HRG (5 ng/ml) or doxorubicin (25 ng/ml) was added directly to the chamber slides, and the cells were incubated at 37°C for 16–18 h fixed and stained for WAF1.

p53 and WAF1 staining

Characterization of the expression of the erbB family receptors and p53 was done immunohistochemically on frozen sections derived from another portion of the same tumor, as previous described (Bacus *et al.*, 1993). Briefly, cells or frozen sections from patients' tumors were fixed for 10 min in 10% neutral buffered formalin (NBF), followed by 3 min in −20°C methanol, and finally 2 min in −20°C acetone. After fixation, the cells were blocked with 10% rabbit serum to which Triton X-100 was added. Primary antibodies to p53 (Mab Pab 1801) or to p21 (Mab EA10) (Oncogene Science, Cambridge, MA); secondary antibody rabbit anti-mouse IgG_1 (Jackson Labs, West Grove, Pa); and tertiary ABC complex (Vectors Labs, Burlington, CA) antibodies were incubated for 30, 20 and 15 min respectively, at 37°C and with PBS washing between incubations. DAB in citrate-phosphate buffer with sodium perborate was used for detection. Cells were counter stained with CAS Ethyl Green (Becton Dickinson, San Jose, CA). Quantitation of the percent of cells expressing p53 and p21 was performed on a dual channel image analysis system as previously described (Bacus *et al.*, 1990).

Cell cycle analysis

Cellular proliferation was assessed by DNA content and incorporation of bromodeoxyuridine (BUdR) by multiparameter flow cytometry (Dolbeare *et al.*, 1983). Cells were plated in growth medium at 2×10^6 per 100 mm plate, allowed to adhere and treated with NDF/HRG (5 or 50 ng/ml) or doxorubicin (25 ng/ml). After 60 h of treatment, BUdR (Sigma, St. Louis, MO) was added to the final concentration of 10 μM and cultures were incubated at 37°C for an additional 60 min. Cultures were then washed twice in PBS to remove floating cells, and the adherent cells were collected after treatment with trypsin. The cells were washed in PBS, fixed in 70% ethanol for 30 min at 20°C, incubated in 1 ml of 0.5% triton X-100 in 2N HCl for 30 min at room temperature, resuspended in 1 ml of 0.1 M $Na_2B_4O_7$, washed and finally incubated with FITC-conjugated anti-BUdR antibody (Becton Dickinson, San Jose, CA) for 30 min at room temperature. The cells were then washed in PBS-Tween-BSA, and resuspended in 1 ml of PBS containing 5 μg/ml of propidium iodide. FACS analysis was performed on a EPICS-753 flow cytometry. The percentage of cells in each phase of the cell cycle (at the end of BUdR labeling period) was estimated using the MDADS program.

RNA Isolation and Northern blot hybridization

Total cellular RNA was extracted from MCF-7, MN1 and MDD2 cells by guanidinium isothiocyanate and subjected to cesium chloride gradient purification. For Northern blot analysis, 20 μg of total RNA was fractionated under denaturing conditions on a 1.2% agarose/0.66 M formaldehyde gel and transferred to a Nytran filter (Schliecher & Schell) for subsequent hybridization. The DNA probes were prepared by random-primed labeling (Boehringer Mannheim). Vector-containing p53 was provided by B Vogelstein, CIP1/WAF1 cDNA was provided by S Elledge and W Harper, and 36B4 cDNA clone was a gift from P Chambon. All cDNA inserts were labeled with [α^{32}P]dCTP to a specific activity of 1×10^9 d.p.m./μg of DNA. Quantitation of autoradiograms was carried out on betascope analyzer (Betagen).

Western blot analysis

Cell lysates were prepared and subjected to Western blot anlaysis as previously described (Keyomarsi *et al.*, 1994). Briefly, 50 μg of protein from each cell line was electrophoresed in each lane of the 13% sodium dodecyl sulfate-polyacrylamide gel (SDS – PAGE) and transferred to Immobilon P (Millipore, Bedford, MA). Blots were incubated with blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% dried milk, 0.2% Tween) overnight at 4°C then further incubated with various primary antibodies diluted in blocking buffer for 3 h. Primary antibodies used were rabbit anti-human p21 or p27 at a dilution of 1 : 1000 (Santa Cruz Biotechnology, Santa Cruz, CA), and antibodies to p53 (Oncogene Science, Cambridge, MA). The blots were then washed and incubated with anti-rabbit horse radish peroxidase conjugate at a dilution of 1 : 5000 in blocking buffer for 1 h, and finally washed and developed with detection reagents (ECL) supplied by Amersham Biochemicals. ECL exposures for all Western blots are of similar duration, i.e., 1–10 s. All quantitation of the Western blot analysis were performed by reprobing the blots with an antibody to actin followed by densitometry. The primary actin antibody used was a mouse monoclonal antibody to actin, clone C4 (Boehringer Mannheim, Indianapolis, IN) at 1 : 500 dilution. Densitometry was performed by scanning of all autoradiographs with AFGA, Arcus II Scanner followed by analysis using NIH Image software.

Immune complex kinase and Western blot assays

Immunoprecipitations and H1 Kinase assays were performed as previously described (Keyomarsi *et al.*, 1995). Briefly, 250 μg of protein were used per immunoprecipitation with polyclonal antibody of CDK2 in lysis buffer containing 50 mM Tris HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 0.5 mM PMSF, 50 mM NaF and 0.5 mM sodium ortho-vanadate. The protein/antibody mixture was incubated with protein A Sepharose for 1 h and the immunoprecipitates were then washed twice with lysis buffer and four times with kinase buffer (50 mM Tris HCl pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA). For H1 kinase assays, the immunoprecipitates were then incubated with kinase buffer containing 5 μg histone H1, 60 μM cold ATP and 5 μCi of [^{32}P]γATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were run on a 13% SDS–PAGE gel. The gel was then stained, de-stained, dried and exposed to X-ray film. For quantitation the protein bands corresponding to histone H1 were excised, and radioactivity was measured by scintillation counting. For IP-Western assays, the immunoprecipitates were electrophoresed on a 13% SDS–PAGE, transferred to Immobilon P (Millipore, Bedford, MA), blocked, and incubated with monoclonal antibody to p21 (Oncogene Science, Cambridge, MA) at 1 : 500 dilution.

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Deregulation of cyclin E in breast cancer

Khandan Keyomarsi, Darryl Conte Jr, Wendy Toyofuku and M Pat Fox

Division of Molecular Medicine, Laboratory of Diagnostic Oncology, Wadsworth Center, Albany, New York 12201, USA

Cyclin E, a regulatory subunit of cyclin dependent kinase-2, is thought to be rate limiting for the G1/S transition during the mammalian cell cycle. Previously, we showed severe alterations in cyclin E protein expression in human mammary epithelial cell lines and in surgical material obtained from patients with various malignancies. To understand the functional basis of these alterations we analyse here the regulation of cyclin E in breast cancer cells. We find that while cyclin E protein and its associated kinase activity in normal cells are cell cycle regulated, in tumor cells it remains in an active complex throughout the cell cycle. We also analysed cyclin E for possible deletions which could result in its constitutive function and found two novel truncated variants in its coding region. These variant forms of cyclin E were detected in several normal and tumor cell lines and tissue specimens. However, Western blot analysis indicated that only the multiple isoforms of cyclin E protein were expressed in tumor but not the normal tissue specimen, suggesting post transcriptional regulation of cyclin E. Lastly, *in vitro* analyses indicated that these truncated variant forms of cyclin E are biochemically active in their ability to phosphorylate histone H1. Collectively these observations suggest the presence of more than one form of cyclin E mRNA in all cells, normal and tumor. Once translated in tumor cells, the protein products of these truncated forms could give rise to a constitutively active form of cyclin E containing complexes.

Keywords: breast cancer; cell cycle; cyclin E; alternative splicing

Introduction

Cyclins are prime cell cycle regulators and central to the control of cell proliferation in eukaryotic cells via their association with and activation of cyclin-dependent protein kinases 1–7 (cdks) (reviewed in, Elledge and Spottswood, 1991; Heichman and Roberts, 1994; Hunter and Pines, 1994; King *et al.*, 1994; Nurse, 1994; Sherr, 1994; Morgan, 1995). Cyclins were first identified in marine invertebrates as a result of their dramatic cell cycle expression patterns during meiotic and early mitotic divisions (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standart *et al.*, 1987; Sherr, 1993). Several classes of cyclins have been described and are currently designated as cyclins A-H, some with multiple members (reviewed in Draetta, 1994). Cyclins can be distinguished on the basis of conserved sequence motifs, patterns of appearance and apparent functional roles during specific phases and regulatory points of the cell cycle in a variety of species.

The connection between cyclins and cancer has been substantiated with the D type cyclins (Hunter and Pines, 1991; Sherr, 1993; Draetta, 1994; Hunter and Pines, 1994). Cyclin D1 was identified simultaneously by several laboratories using independent systems: It was identified in mouse macrophages due to its induction by colony stimulating factor 1 during G1 (Matsushime *et al.*, 1991); in complementation studies using yeast strains deficient in G1 cyclins (Lew *et al.*, 1991; Xiong *et al.*, 1991); as the product of the *bcl*-1 oncogene (Withers *et al.*, 1991) and as the PRAD1 proto-oncogene in some parathyroid tumors where its locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene (Matsushime *et al.*, 1991; Motokura *et al.*, 1991, Motokura and Arnold, 1993; Quelle *et al.*, 1993). In centrocytic B cell lymphomas cyclin D1 (PRAD1)/BCL1 is targeted by chromosomal translocations at the BCL1 breakpoint, t(11;14) (q13;q32) (Rosenberg *et al.*, 1991a,b). Furthermore, the cyclin D1 locus undergoes gene amplification in mouse skin carcinogenesis, as well as in breast, esophageal, colorectal and squamous cell carcinomas (Lammie *et al.*, 1991; Jiang *et al.*, 1992, 1993b; Bianchi *et al.*, 1993; Buckley *et al.*, 1993; Leach *et al.*, 1993). Several groups have examined the ability of cyclin D1 to transform cells directly in culture with mixed results (Hinds *et al.*, 1992, 1994; Jiang *et al.*, 1993a; Quelle *et al.*, 1993; Rosenwald *et al.*, 1993; Sherr, 1993; Lovec *et al.*, 1994; Musgrove *et al.*, 1994; Resnitzky *et al.*, 1994). However, the overexpression of cyclin D1 was recently observed in mammary cells of transgenic mice and results in abnormal proliferation of these cells and the development of mammary adenocarcinomas (Wang *et al.*, 1994). This observation strengthens the hypothesis that the inappropriate expression of a G1 type cyclin may lead to loss of growth control.

Recently, we and others have reinforced the linkage between oncogenesis and the cell cycle by correlating the deranged expression of cyclins to the loss of growth control in breast cancer (Buckley *et al.*, 1993; Keyomarsi and Pardee, 1993). Using proliferating normal *vs* human tumor breast cell lines in culture as a model system, we have described several changes that are seen in all or most of these lines. These include increased cyclin mRNA stability, resulting in overexpression of mitotic cyclins and cdc2 RNAs and proteins in 9/10 tumor lines, leading to the deranged order of appearance of mitotic cyclins prior to G1 cyclins in synchronized tumor cells.

The most striking abnormality in cyclin expression we found, was that of cyclin E. Cyclin E protein not only was overexpressed in 10/10 breast tumor cell lines but it was also present in lower molecular weight

Correspondence: Khandan Keyomarsi
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isoforms than that found in normal cells (Keyomarsi and Pardee, 1993). We directly examined the relevance of cyclin derangement to *in vivo* conditions, by measuring the expression of cyclin E protein in tumor samples *vs* normal adjacent tissue obtained from patients with various malignancies (Keyomarsi *et al.*, 1994). These analyses revealed that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alteration in cyclin E protein expression independent of the S-phase fraction of the samples. In addition, the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as PCNA or c-*erb*B2. These observations strongly suggested the use of cyclin E as a new prognostic marker.

In this report, we have further characterized the alterations of cyclin E in breast cancer. We show that while cyclin E is cell cycle regulated in normal cells it is present constitutively and in an active cdk2 complex in synchronized populations of breast cancer cells. We also identify two novel truncated variant forms of cyclin E mRNA as detected by RT-PCR which are ubiquitously detected in normal and tumor cells and tissues. These variant forms of cyclin E can give rise to an active cyclin/cdk2 complex *in vitro*, but they do not seem to be translated in normal cells.

Results

Elevated cyclin E associated kinase activity in breast cancer cells

To test the hypothesis that the altered expression pattern of cyclin E protein found in tumor cell lines and tissue samples (Keyomarsi *et al.*, 1994) is associated with increased cyclin E kinase activity, we compared cyclin E expression and activity in two normal *vs* five breast cancer cell lines (Figure 1). The two normal cell lines are the normal cell strain, 76N (Figure 1, lane 1), obtained from reduction mammoplasty and a near diploid immortalized cell line MCF-10A (Figure 1, lane 2) (Soule *et al.*, 1990). 76N is a mortal cell strain since it rapidly proliferates (doubling time of 24–27 h) for multiple passages before senescence at around passage 20 (Band and Sager, 1989). The MCF-10A cell line is a spontaneously immortalized human breast epithelial cell line which can be cultured indefinitely. This cell line has no tumorigenicity potential but retains characteristics of a normal breast epithelial cell line (Soule *et al.*, 1990).

We examined the pattern of cyclin E protein expression in normal *vs* tumor cell lines using monoclonal and polyclonal antibodies to cyclin E on Western blots (Figure 1A). Similar immunoblot banding patterns were obtained with either the monoclonal or polyclonal antibody to cyclin E, confirming the specificity of the multiple bands. However, the patterns of cyclin E protein expression was different between normal and tumor cells. Both cyclin E antibodies recognized one major protein migrating at ~50 kDa and two much less abundant lower molecular weight forms, in the two normal cell lysates. In the tumor cell lysates on the other hand, the

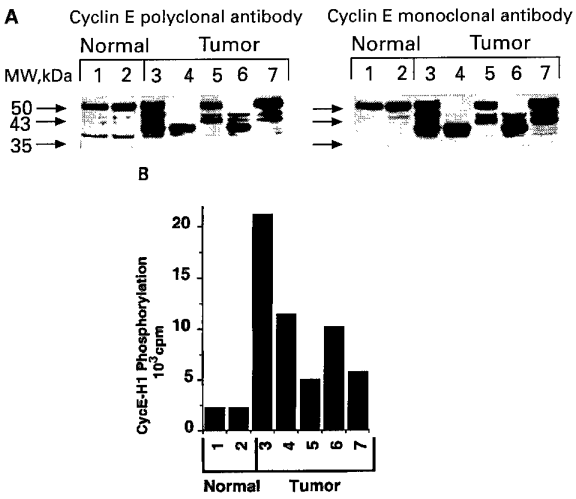

Figure 1 Correlation of cyclin E protein(s) to cyclin E associated kinase activity (**A**) Western blot analysis of cyclin E expression in normal *vs* tumor cells using two different cyclin E antibodies. Whole cell lysates were extracted from the seven cell lines, (100 µg of protein extract/lane), run on a 10% acrylamide gel and blotted as described in Materials and methods. Lane 1, 76N normal human mortal breast epithelial cell strain; lane 2, MCF-10A normal immortalized human breast epithelial cell line; (lanes 3–7 are all human breast cancer cell lines) lane 3, MDA-MB-157; lane 4, MDA-MB-436; lane 5, ZR75T; lane 6, SKBR3; lane 7, MCF-7. The 50 kDa arrowhead points to the cyclin E protein of the predicted size. The other arrowheads point to the additional cyclin E isoforms observed in the tumor cell lines ranging in molecular weight from 35 to 43 kDa. Molecular mass standards were used in each gel to estimate the position of each band. (**B**) Cyclin-E associated histone H1 kinase activity. Equal amounts of proteins from cell lysates were immunoprecipitated with anti-cyclin E coupled to protein A beads using histone H1 as substrate. The associated kinase activities were quantified by scintillation counting

same antibodies recognized three (lane 3), two (lanes 5–7) or one (lane 4) additional and highly abundant isoforms of cyclin E protein that in each case revealed a different pattern from that of the normal cells.

We next analysed the cyclin E associated protein kinase activity in all cells by measuring the phosphorylation of histone H1 in immunoprecipitates made with the polyclonal antibody to cyclin E (Figure 1B). In all of the tumor cell lysate immunoprecipitates, the activity levels of cyclin E-associated kinase were significantly higher than that of both normal cells. For example, in MDA-MB-436 and SKBR3 tumor cell lines (lanes 4 and 6) which express only the lower molecular weight isoforms of cyclin E protein, the associated kinase activity was sixfold greater than that of the normal cells which express mainly the high molecular weight, 50 kDa, form of cyclin E protein. Similarly, the other tumor lines containing altered patterns of cyclin E expression, had significantly higher cyclin E-associated H1-kinase activity as compared to the normal cell strains.

Lack of cell cycle regulation of cyclin E in breast cancer cells

In one tumor line, MDA-MB-157 (Figure 1, lane 3), the level as well as the associated kinase activity of cyclin E protein was the highest of all the tumor cell lines examined. Previous studies (Keyomarsi and

Pardee, 1993) showed that this overexpression is in part due to an eightfold amplification of the cyclin E gene and 64-fold overexpression of its mRNA in this cell line. The cyclin E gene is amplified in tandem and is not associated with gross genomic rearrangements (data not shown). To investigate whether the signals required for normal regulation of cyclin E expression are altered or lost in tumor cells, the cell cycle expression of cyclin E protein and its associated kinase activities in the MDA-MB-157 cell line were compared to normal mammary epithelial 76N cells (Figure 2).

Both cell lines were synchronized in the G1/S border by double thymidine block. Synchrony of both cell types at several times after release from the block was monitored by flow cytometry * (Figure 2D). At various times after release from treatment for synchronization, cells were harvested and extracted proteins were analysed on Western blots with antibodies to cyclins E and A (Figure 2A). In normal 76N cells, the pattern of expression of cyclin E and cyclin A proteins is consistent with that seen for other normal cell types with levels rising prior to S phase and oscillating thereafter in the cell cycle (Koff *et al.*, 1992). In addition there is only one major form (i.e., 50 kDa) of cyclin E protein detected and there is a shift in the timing of when cyclin E *vs* cyclin A appears in the cell cycle of these normal epithelial cells. However, in the tumor cells, cyclin E protein does not appear to be cell cycle regulated and multiple isoforms of the protein are also present with similar signal intensities and banding patterns during the time intervals examined. In addition when these tumor cells are synchronized by other agents, such as Lovastatin (Keyomarsi *et al.*, 1991), cyclin E expression is also constitutive throughout the cell cycle, resembling a pattern identical to that shown in Figure 2A (data not shown). In the same tumor cell extracts, cyclin A protein is cell cycle regulated with peak levels coinciding with peak S and early G2/M phase. Hence, it appears that in this tumor cell line, cyclin E is abnormally regulated during the cell cycle.

In order to compare the kinase activity associated with cyclin E and cdk2 in normal and tumor cells, we measured the phosphorylation of histone H1 in immunoprecipitates prepared from synchronous cell extracts using antibody to either cyclin E or cdk2 (Figure 2B). There were two significant differences found between normal and tumor cells: First, in the length of time which an active cyclin E/cdk2 complex is present and secondly in the amount of kinase activity associated with cyclin E *vs* cdk2 during the normal and tumor cell cycles. In normal cells, both cyclin E associated kinase and cdk2 activities are cell cycle regulated, coinciding with the levels of cyclins E and A protein expression (Figure 2A). In addition, the cdk2 activity is one order of magnitude (i.e. 10-fold) higher than cyclin E associated activity, consistent with cdk2's ability to form an active complex with other cyclins besides cyclin E in normal cells (Figure 2B). Hence, cyclin E in these normal cells is indeed cell cycle regulated and the signals required for such regulation are intact both at the protein expression level and kinase activity.

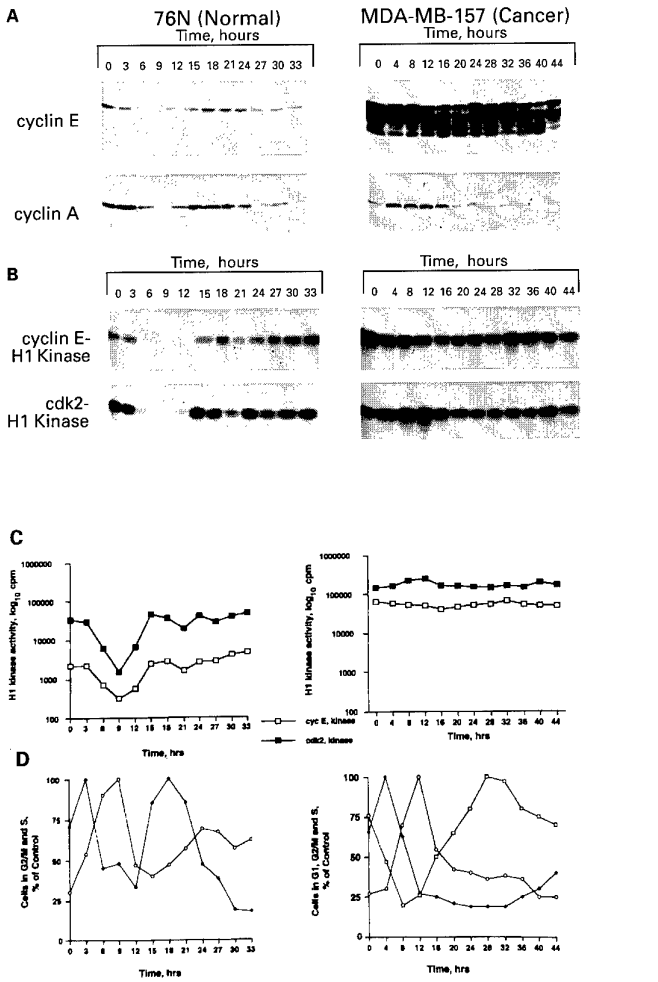

Figure 2 Expression of cyclin E in synchronized normal 76N and tumor MDA-MB-157 breast cells. Both cell types were synchronized by double thymidine block procedure (see Materials and methods). At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to (**A**) Western blot and (**B**) Histone H1 kinase analysis. Protein (50 μg) for each time point was applied to each lane of a 10% acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity purified polyclonal antibodies. The blots were stripped between the two assays in 100 mM β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8) and 2% SDS for 30 min at 55°C. For Histone H1 kinase activity, equal amount of proteins (600 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) or anti-CDK2 (polyclonal) coupled to protein A beads using histone H1 as substrate. Panel B is the autoradiogram of the histone H1 SDS–PAGE gel and (**C**) shows the quantification of the histone H1 associated kinase activities by scintillation counting. Open symbols correspond to cyclin E associated kinase activity and closed symbols correspond to cdk2 activity (**D**). At various times after release from double thymidine block, aliquots were removed and subjected to flow cytometry analysis. Cells in S phase (◆), G2/M phase (○) and G1 phase (□), are expressed as percent of control, where control is equal to the time when the maximum number of cells enter each phase of the cell cycle

*The doubling times of the normal 76N and tumor MDA-MB-157 cells are 27 and 36 h, respectively, and their DNA content distribution in different cell cycle phases are as follows: 76N- G1 (75%), S (4%) and G2/M (21%); MDA-MB-157-G1 (56%), S (13%) and G2/M (31%).

In tumor cells, on the other hand, cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle resulting in a constitutive pattern of histone H1 phosphorylation. The basal levels of cyclin E associated kinase activity during the tumor cell cycle, at any time interval examined, are at least 20 times higher than that of the normal cells (Figure 2C). Cdk2, a kinase which binds to both cyclin E and A, is also constitutively active during the cell cycle. However, cdk2 activity in this tumor cell line is only twofold higher than cyclin E associated kinase activity, presumably due to the abundance of cyclin E protein which is capable of sequestering cdk2. When cyclin A protein levels are induced in the tumor cells, there is only a 30% additional induction in cdk2 associated activity. These observations suggest that cyclin E protein, which is constitutively expressed in the cell cycle of tumor cells, also results in an active kinase complex throughout the cell cycle. Furthermore, since the same cyclin-dependent kinase can be regulated by both cyclins E and A, increased levels of cyclin E may overcompensate for cyclin A regulation, again resulting in a constitutively active and abundant cyclin E/cdk2 complex.

Isolation of variant forms of cyclin E transcripts

In an attempt to determine the presence of any potential alterations in the cyclin E gene in MDA-MB-157, we amplified the entire cyclin E coding region of this cell line by reverse transcription-polymerase chain reaction amplification (RT-PCR), cloned these products and analysed their DNA sequence (Figure 3). Using a pair of primers flanking the coding sequence of cyclin E gene, we observed at least two distinct PCR products ranging in size from 1.0 to 1.2 kb from the MDA-MB-157 RT template (Figure 3A). The product from the control (cyclin E plasmid DNA) was of 1.2 kb, corresponding to the full length cyclin E cDNA isolated from a HeLa cDNA library (Koff *et al.*, 1991; Lew *et al.*, 1991). We cloned the RT-PCR products from the MDA-MB-157 cell line and confirmed their identity by Southern blotting and by DNA sequencing (data not shown). Three independent RT-PCR reactions were performed on freshly isolated RNA from this cell line. Fifteen clones from each RT-PCR reaction were examined further. Sequence analyses revealed two types of truncated variants of the cyclin E gene, as well as an unequivocally normal sequence, from the MDA-MB-157 cell line (Figure 3).

The PCR products containing these two truncated variants were termed cyclin E-Δ9 and cyclin E-Δ148 (Figure 3B). The alteration in clone cyclin E-Δ9 is a 9 base pair in-frame deletion of nucleotides 67–75 at the 5′ end of the gene, while the alteration in clone cyclin E-Δ148 is a 148 base pair deletion of nucleotides 1000–1147 at the 3′ end of the gene resulting in a frame shift transcript. Curiously, the 148 bp deletion in cyclin E-Δ148 clone disrupts the PEST sequence motif of the gene, which is thought to be important for its role in degradation of the protein product (Koff *et al.*, 1991; Lew *et al.*, 1991). The relative positions of these two newly identified truncations to the wild type sequence of cyclin E are shown in Figure 3B. We performed *in vitro* translation studies on these clones

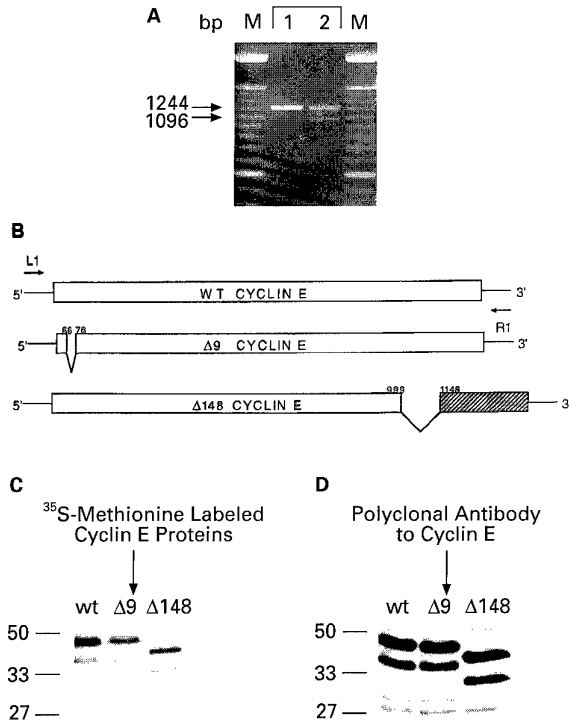

Figure 3 Identification and *in vitro* translation of cyclin E truncated transcripts. (**A**) PCR amplified cyclin E coding sequence using primers (L1CYCE and R1CYCE) flanking the entire coding region of cyclin E. Lane 1: Molecular weight standards; lane 2: control template DNA, a plasmid containing a wild type cyclin E coding sequence; lane 3: RT-PCR amplification of cyclin E using RNA from MDA-MB-157; lane 4: Molecular weight standards. PCR conditions were carried out as described in Materials and methods. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Molecular weight markers in base pairs are indicated (left). (**B**) Relative position of cyclin E Δ9 and Δ148 deletions to the wild type cyclin E sequence. The two arrows flanking the cyclin E coding region refer to the position of R1 (i.e., R1CYCE) and L1 (i.e.,L1CYCE) oligonucleotides used for the RT-PCR reactions. (**C**) The cDNAs of cyclin E clones were subcloned into PCR II vector and transcribed and translated *in vitro* using T7 RNA polymerase-rabbit reticulocyte lysate system in the presence of [^{35}S]methionine and products were analysed on a 10% SDS–PAGE followed by autoradiography (**D**) The *in vitro* translated cyclin E protein products from the three different clones (in the absence of radioactivity) were subjected to Western blot analysis and hybridized to a polyclonal antibody to cyclin E

using T7 RNA polymerase (Figure 3C and D). RNA was translated in the presence of [^{35}S]methionine using a rabbit reticulocyte lysate, analysed by SDS–PAGE and visualized by autoradiography. Cyclin E-wt and cyclin E-Δ9 protein products showed very similar electrophoretic mobilities (Figure 3C). [On a sequencing length SDS–PAGE, gel however, we were able to detect the slight (3 amino acids) molecular weight difference between the two clones (data not shown)]. Cyclin E-Δ148 gives rise to a protein product which is ~5 kD smaller than the cyclin E-wt, which would correspond to the loss of the 50 amino acids. To confirm that the protein products from *in vitro* translation reactions were indeed cyclin E, the cDNAs of the three different clones were transcribed and translated in the presence of unlabeled methionine and the products were subjected to Western blot analysis

(Figure 3D). The protein products from cyclin-wt, Δ9 and Δ148 clones reacted strongly with the polyclonal antibody to cyclin E, suggesting that the *in vitro* translated products of these clones are truncated forms of cyclin E. Interestingly, all the clones gave rise to two major protein products, migrating at ~45 and ~38 kD for cyclin E-wt and Δ9 clones and ~40 and ~33 kD for cyclin E-Δ148 clone. It is not clear at this point whether the lower molecular weight protein product is a result of proteolytic cleavage, or result of translation initiation from a methionine site further downstream in the coding region.

Expression of cyclin E truncated variants in normal vs *tumor cells and tissue samples*

Since these two truncated forms of cyclin E cDNA were isolated from one tumor-derived cell line, we investigated the generality of expression of the cyclin E variants in a panel of 13 breast epithelial cell lines (Figure 4A). These cell lines included three normal mortal cell strains (lanes 1–3), one normal immortalized cell line (lane 4) and nine tumor-derived breast cell lines (lanes 5–13). These analyses revealed the presence of multiple transcripts of cyclin E in all cell lines examined. However, no distinct differences were observed in their pattern of expression between normal *vs* tumor cell lines. Furthermore, sequence analysis of a cloned RT-PCR product of 76N normal cells revealed that the major transcript found in this normal cell strain is the Δ148 variant of cyclin E previously identified in MDA-MB-157 tumor cell line (data not shown). These observations indicate that the Δ148 RNA is expressed in all cells examined at an apparently higher level than the wild type species of cyclin E RNA. To examine the specific expression of Δ9 and Δ148 in each cell line, we performed RT-PCR using primers that spanned the deleted sequences, such that only those cell lines containing cyclin E transcripts harboring these deletions would give rise to products. These analyses show that the Δ9 variant form of cyclin E is abundantly present in three cell lines, two of which are normal cell strains and one is the MDA-MB-157, the original cell line this variant form was isolated from (Figure 4B). In addition we find that the Δ148 is present in all cell lines examined (Figure 4C), confirming our previous observation that this variant form of cyclin E is the major transcript found in these cells (Figure 4A).

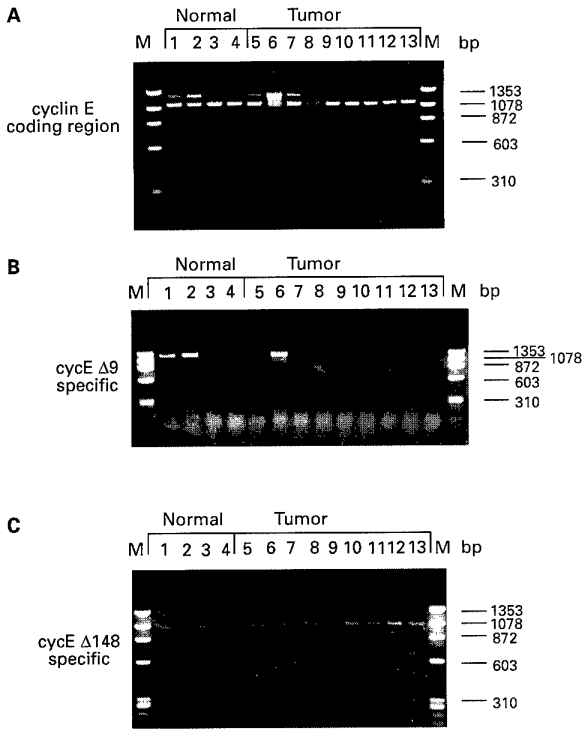

Figure 4 RT-PCR amplification of cyclin E Δ9 and Δ148 in normal and tumor-derived breast epithelial cell lines. RT-PCR amplification of cyclin E coding sequence from normal and tumor-derived breast epithelial cell lines using (**A**) primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E and amplifying wild type cyclin E sequences, as well as those containing internal deletions, (**B**) primers (LMEMARK3 and R1CYCE) spanning the Δ9 deletion and amplifying only those cyclin E sequences harboring the Δ9 internal deletion of cyclin E and (**C**) primers (L1CYCE and RMEMARK3) spanning the Δ148 internal deletion and amplifying only those cyclin E sequences containing the Δ148 deletion. The cell lines used are as follows: Lane 1, 70N; lane 2, 81N; lane 3, 76N; lane 4, MCF-10A; lane 5, MCF-7; lane 6, MDA-MB-157; lane 7, MDA-MB-231; lane 8, MDA-MB-436; lane 9, T47D; lane 10, BT-20T; lane 11, HBL-100; lane 12, Hs578T and lane 13, ZR75T. Normal cells are represented in lanes 1–4 and tumor-derived cell lines in lanes 5–13; M, Molecular weight size markers

In order to apply our findings from culture studies to the *in vivo* condition, we examined whether the truncated cyclin E transcripts were also expressed in tumor tissue specimens. We performed RT-PCR using RNA isolated from seven paired samples of human breast carcinoma and normal adjacent tissue (NAT) which are presented according to increased clinical stage (Figure 5). For this experiment, we used primers flanking the entire coding region of cyclin E in order to detect all variants of cyclin E which could contain deletions in the coding region. The RT-PCR products from NAT and tumor tissue samples ranged in size from 1.0 to 1.2 kb (Figure 5A), which are consistent with products obtained with cultured breast cells (Figure 4). Surprisingly, we found that not only did both NAT and tumor tissue samples express similar RT-PCR products corresponding to the cyclin E variants, but that no distinct difference could be found among paired samples as the clinical stage of the disease increases. On the other hand, when we subjected whole cell lysates prepared from these tissue specimens to Western blot analysis, we did observe cyclin E protein alterations which increased qualitatively and quantitatively as the stage of the disease increased. In high staged tumor samples, an antibody to cyclin E reacted strongly with at least three overexpressed proteins ranging in size from 35 to 50 kDa, while in the NAT samples, one major protein of 50 kDa was present at very low levels, consistent with our previous observations (Keyomarsi *et al.*, 1994). Collectively these observations suggest that at the level of RNA there are no apparent differences between normal and tumor cells or between tissue samples in their ability to express the alternate transcripts of cyclin E. However, the alteration in cyclin E protein observed exclusively in tumor cells,

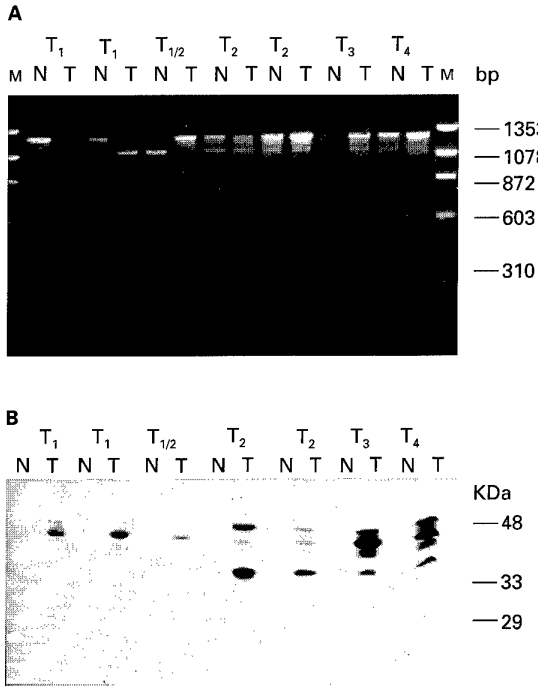

Figure 5 Comparison of RT-PCR amplified products of cyclin E with their expression in breast cancer specimens. (**A**) RT-PCR amplification of cyclin E coding sequence using total RNA isolated from seven pairs of normal adjacent (NAT) and tumor tissue samples with primers (L1CYCE and R1CYCE) flanking the coding region of cyclin E as described in Figure 4A. (**B**) Western blot analysis of whole cell lysates (100 μg) were prepared from NAT and tumor tissue specimens and probed with a monoclonal antibody to cyclin E. Breast cancer types and histological/tumor grades are as follows: Lanes 1–2, intraductal carcinoma of the breast, Stage T_1, NO, MO, Grade I; Lanes 3–4, invasive well differentiated ductal carcinoma, Stage T_1, NO, MO, Grade I; Lanes 5–6, intraductal carcinoma, Stage $T_{1/2}$, NO, MO, Grade I; Lanes 7–8, invasive and intraductal carcinoma, Stage T_2, NO, MO, Grade II; Lanes 9–10, *in situ* and infiltrating ductal carcinoma, Stage T_2, N1, MO, Grade II/III; Lanes 11–12, infiltrating ductal carcinoma, Stage T_3, NO, MO, Grade II/III; Lanes 13–14, invasive ductal carcinoma, Stage T_4, NO, MO, Grade III. Molecular mass standards were used on each gel to estimate the position of each band

likely occurs post transcriptionally or translationally to result in various forms of the protein detected in tumor but not normal cells or tissues.

Cyclin E truncated variants form biochemically active complexes with cdk2

Based on the evidence that multiple cyclin E transcripts (Figures 4 and 5) are found in normal and tumor cells as well as in tissue samples and that there is an active cyclin E/cdk2 protein complex present throughout the cell cycle of the MDA-MB-157 cell line (Figure 2), we asked whether these alternate transcripts of cyclin E can give rise to a biochemically active product. To investigate this question, we overexpressed cyclin E and cdk2 in insect cells using the baculovirus expression system (Figure 6). Insect cells were co-infected with the recombinant baculovirus containing cdk2 and either cyclin E- wild type (cycE-wt), cyclin E-Δ9, (cycE-Δ9), or cyclin E-Δ148 (cycE-Δ148) cDNAs (Figure 6). At the indicated times (i.e. days) following infection, cell extracts were collected, homogenized and subjected to Western blot and histone H1 kinase analysis. Western blot analysis shows that there were similar levels of expression of the three cyclin E variants and cdk2 in the infected sf9 cells within one day of infection and thereafter during the course of experiment (Figure 6A). H1 kinase analysis reveal that when the cyclin E-wt/cdk2 co-infected insect cell lysates were immunoprecipitated with an antibody to cdk2, the immunoprecipitates were capable of phosphorylating histone H1 within one day of infection and an active cyclin E/cdk2 complex persisted throughout the experiment (Figure 6B, lanes 1–4). In insect cells co-infected with the two truncated variants of cyclin E, similar results were obtained illustrating that the complex which cycEΔ9 (Figure 6C, lanes 5–8) or cycEΔ148 (Figure 6C, lanes 9–12) formed with cdk2 is also active and is capable of phosphorylating histone H1. However a lower degree of activation was found compared to that with the cyclin E wild type complex. There was a twofold difference in the ability of the cycEΔ9/cdk2 or cycEΔ148/cdk2 to phosphorylate histone H1 when

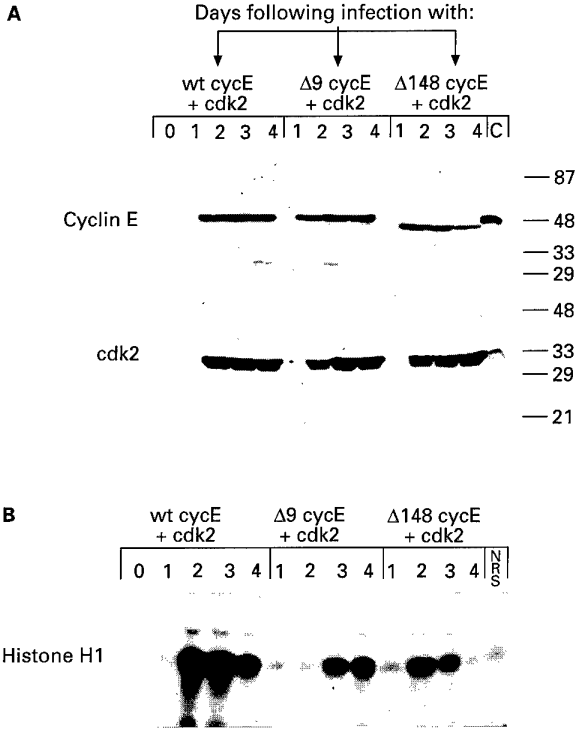

Figure 6 Activation of cdk2 by cyclin E wild type and its truncated variants in insect cells. Cell lysates were prepared from insect cells co-infected with baculovirus containing the different cyclin E constructs and cdk2 at the indicated time intervals (days) following co-infection. (**A**) Equal amounts (50 μg) of protein were added to each lane; the gel was then subjected to Western blot analysis with polyclonal antibody to cyclin E or to cdk2. C: Control lane corresponding to 50 μg of extracts from insect cells infected with either cyclin E wt alone, or cdk2 alone baculovirus (**B**) Histone H1 kinase assays were also performed on the same cell extracts by immunoprecipitating equal amount of cell lysate with polyclonal antibody to cdk2 coupled to protein A beads using histone H1 as substrate. The autoradiogram of the histone H1 SDS–PAGE gel is depicted. NRS: A control immunoprecipitate performed with *N*ormal *R*abbit *S*erum in place of cdk2 antibody

compared to cyclin E wt/cdk2. These *in vitro* analyses suggest that once the cyclin E variant transcripts are translated, the protein products can give rise to a functionally active cyclin E complex capable of phosphorylating substrates such as histone H1.

Discussion

In an attempt to understand the relationship between the cell cycle and cancer, many laboratories have investigated the role cyclin/cdk complexes play in cancer. While cyclins D and A have been implicated in tumorigenesis, the role of other cyclins have been elusive and limited mainly to observations. Cyclin E is an interesting case since it shows an altered pattern of expression in all breast cancer cell lines and tumor tissue samples we have examined to date (Keyomarsi *et al.*, 1994). The cyclin E alterations include over-expression of the authentic-sized protein as well as expression of lower molecular weight isoforms found in tumor cells or tissues. We set out to decipher the mechanism responsible for these alterations by initially correlating the activity of cyclin E/cdk2 complexes with the expression pattern and level of cyclin E protein, both in exponentially growing and synchronized population of normal *vs* tumor cells. We find that regardless of which combination of the cyclin E (50 kDa) and its lower molecular weight isoforms are expressed in these tumor cell lines, the associated kinase activity is much higher in tumor than normal cells. Furthermore, we find that in synchronized populations of tumor cells, cyclin E is present in altered forms throughout the cell cycle and the kinase activity associated with it, or with cdk2, is also constitutively active. In addition the abundant and constitutive expression of cyclin E in these tumor cells result in sequestering of cdk2 away from other cyclins, such as cyclin A. This suggests that, there is a cyclin E/cdk2 complex which is abundantly and uniformly active in the tumor but not the normal cell cycle.

In order to determine whether the multiple forms of the cyclin E protein detected in tumor cells originate from different transcripts of cyclin E RNA, we performed RT-PCR and found two different truncated variants of cyclin E (i.e. Δ9 and Δ148) expressed in MDA-MB-157 tumor cell line. Further analysis revealed that the most intriguing feature of the Δ9 and Δ148 variant forms of cyclin E is that there is no distinct difference in their mRNA expression in normal *vs* tumor cells or tissue samples. In addition there is little correlation between expression of these cyclin E variants at the level of RNA *vs* protein. Yet, we show here that at the level of protein in tumor cells, (a) cyclin E isoforms ranging in size from 35 to 50 kDa are abundantly expressed (Figures 1, 2 and 5) and that (b) these protein isoforms of cyclin E are not subject to cell cycle regulation and may constitutively interact with cdk2 resulting in an active complex (Figure 2). Lastly (c) we also show that once Δ9 and Δ148 transcripts of cyclin E are allowed to express their protein products, the resulting proteins can bind to cdk2 and form active complexes *in vitro* (Figure 6). Collectively, based on these observations we suggest that the multiple protein isoforms of cyclin E detected in tumor cells are a result of altered post-transcriptional and/or translational regulation of cyclin E mRNAs. Hence, there may be a translational fidelity that has been altered/lost in tumor cells, allowing for the translation of these truncated variants of cyclin E to occur and once translated, they can form active complexes with cdk2 throughout the cell cycle (see Figures 2 and 6). Alternatively, there may be post-translational modification of cyclin E which is also altered or lost in tumor cells. The presence of lower molecular weight protein isoforms of cyclin E, barely detectable in normal cell lysates (Figure 1, lanes 1 and 2) could also suggest that these isoforms of cyclin E are in fact translated in normal cells as well, but they are rapidly degraded. In tumor cells, the protein turnover is much longer and as a result we can readily detect lower molecular weight isoforms of cyclin E which are highly abundant and functionally active.

One possibility for the presence of multiple transcripts of cyclin E is due to alternative splicing. Precedent for alternative splicing of cyclin E has recently been reported by Ohtsubo *et al.* (1995) where they identified a longer form of cyclin E (cyclin E-L) which contains 15 amino acids at the amino terminus which through alternative splicing, is absent in the original form of cyclin E (cyclin E wt) (Ohtsubo *et al.*, 1995). In addition Sewing *et al.* (1994) also identified another splice variant of cyclin E, termed cyclin Es. Like cyclin E-L and Es, there is a strong possibility that both cyclin EΔ9 and cyclin EΔ148 reported here, are results of alternative splicing as we find potential splice donor and acceptor sites at the deleted junctions of each transcript. However, the cyclin Es variant differs from those we report here in that cyclin Es lacks 49 amino acids within the cyclin box and is 90% less abundant than the wild type cyclin E sequence. This form is unable to associate with cdk2, is inactive in histone H1 kinase assays and is unable to rescue a triple CLN mutation of *S. cerevisiae* (Sewing *et al.*, 1994). Unlike cyclin Es, neither the cyclin EΔ9 nor the Δ148 transcripts disrupts the cyclin box, the consensus region which confers activity by its association to a cdk (Lees *et al.*, 1992). As a result, both Δ9 and Δ148 variants of cyclin E retain the ability to functionally bind to cdk2 and phosphorylate histone H1 in insect cells (Figure 6). The ability of these novel variants of cyclin E to form an active complex with cdk2 has implications for their biological functions. The Δ148 variant has another interesting feature in that the PEST sequence important for its degradation has been disrupted by this 148 base pair deletion. It is possible that the deletion of a PEST sequence may have an effect on turnover of Δ148 cyclin E, allowing it to remain active for a longer duration than the wild type form.

The data presented here suggest that the mechanisms responsible for the presence of the multi isoforms of cyclin E protein in tumor cells may be due to a number of factors, one of which is the altered post-transcriptional or translation regulation of the truncated variants of cyclin E. However a question can be raised whether these two novel variant forms of cyclin E attribute to the cancer phenotype. We present data that these two variants are not a result of deletional mutations in the cyclin E gene as they are expressed in both normal and tumor cells as well as tissue samples. However, they are not readily detected in normal cells

either due to their lack of translation or rapid degradation. There is evidence that when the wild type cyclin E is overexpressed in normal cells the length of G1 is decreased, but cells are not transformed (Ohtsubo and Roberts, 1993; Resnitzky *et al.*, 1994). With the discovery of the cyclin E variants that may be translated in tumor but not normal cells, the oncogenecity of these cyclin E forms can now be directly deciphered. A second question that our data has raised, is whether the lower molecular weight isoforms of cyclin E detected mainly in tumor cells are the protein products of the cyclin EΔ9 and/or Δ148 variant transcripts of cyclin E. By identification of these two variants, we can now utilize them as molecular probes to identify their protein products in tumor cells and tissues. Identification of the multiple protein isoforms of cyclin E will give us insight as to the regulation of this protein, which when complexed with cdk2 is thought to be rate limiting for the G1/S transition during the mammalian cell cycle. With an active cyclin E/cdk2 complex, substrates may be phosphorylated at altered points in the cell cycle resulting in loss of checkpoint control during the progression of G1 to S in tumor cells.

Materials and methods

Cells lines, culture conditions and tissue samples

The culture conditions for 70N, 81N and 76N normal cell strains and MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-436, T47D, BT-20, HBL100, Hs578T, SKBR3 and ZR75T tumor cell lines were described previously (Keyomarsi and Pardee, 1993). MCF-10A is a normal human mammary epithelial cell line which is spontaneously immortalized and does not grow in soft agar and is not tumorigenic in nude mice (Soule *et al.*, 1990). This cell line was obtained from ATCC and is cultured in DFCI-1 (Band and Sager, 1989). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO_2 and maintained free of Mycoplasma as determined by the MycoTect Kit (Gibco). Snap frozen surgical specimens from patients diagnosed with breast cancer were obtained from the National Disease Research Interchange/Co-operative Human Tissue Network, Eastern Division. The clinical stage and grade of the tissue samples used were obtained from pathology/surgical reports and indicated in the figure legend.

Synchronization and flow cytometry

76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were synchronized at the G1/S boundary by a modification of the double thymidine block procedure (Rao and Johnson, 1970). Briefly, 48 h after the initial plating of cells, the medium was replaced with fresh medium containing 2 mM thymidine for either 24 h (76N cells) or for 36 h (MDA-MB-157 cells). This medium was then removed, the cells were washed three times and subsequently incubated in fresh medium lacking thymidine for 12 h (76N cells) or 24 h (MDA-MB-157 cells). Next cells were re-incubated in medium containing 2 mM thymidine, as above, washed with fresh medium and incubated in thymidine free medium for the rest of the experiment. Cells were harvested at the indicated times, cell density was measured electronically using a Coulter Counter (Hialeah, Florida) and flow cytometry analysis was performed. For flow cytometry studies, 10^6 cells were centrifuged at $1000 \times g$ for 5 min, fixed with ice-cold 70% ethanol (30 min at 4°C) and washed with phosphate buffered saline (Crissman and Tobey 1974). Cells were suspended in 5 ml of phosphate-buffered saline containing 10 μg ml^{-1} RNase, incubated at 37°C for 30 min, washed once with phosphate buffered saline and resuspended in 1 ml of 69 μM propidium iodide in 38 mM sodium citrate. Cells were then incubated at room temperature in the dark for 30 min and filtration through a 75 mm Nitex mesh. DNA content was measured on a FACScan flow cytometer system (Becton Dickinson, San Jose, CA) and data were analysed using CELLFIT software system (Becton Dickinson).

Western blot and H1 kinase analysis

Cell lysates and tissue homogenates were prepared and subjected to Western blot anaylsis as previously described (Keyomarsi and Pardee, 1993; Keyomarsi *et al.*, 1994). Briefly, 100 μg of protein from each tissue sample or cell line (for sf9 extracts, 50 μg) were electrophoresed in each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) (cyclin E and cyclin A), or a 13% SDS–PAGE (cdk2 and all Sf9 cell extracts) and transferred to Immobilon P. Blots were blocked with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% dried milk, 0.2% Tween overnight at 4°C and were incubated with various primary antibodies diluted in blocking buffer for 3 h. Primary antibodies used were rabbit anti-human cyclin E serum at a dilution of 1:2500 (gift from A Koff and J Roberts, Fred Hutchinson Cancer Research Center), monoclonal antibody HE12 to cyclin E at a dilution of 1:10 (a gift of E Lees and E Harlow, Massachusetts General Hospital [MGH] Cancer Center), affinity-purified rabbit anti-human $p33^{cdk2}$ kinase antibody at a dilution of 1:2000 (a gift from L-H Tsai and E Harlow, MGH Cancer Center) and affinity-purified rabbit anti-human cyclin A antibody at a dilution of 1:20 000 (a gift from JW Harper, Baylor College of Medicine). Following primary antibody incubation, the blots were washed and incubated with either goat anti-mouse or anti-rabbit horseradish peroxidase conjugate at a dilution of 1:5000 in blocking buffer for 1 h and finally washed and developed with detection reagents (ECL) supplied by Amersham biochemicals. ECL exposures for all Western blots are of similar duration, i.e. 1–10.

For H1 kinase assays, 250 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with either polyclonal antibody to cyclin E or CDK2 in lysis buffer containing 50 mM Tris HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 25 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 1 mM benzamidine, 10 μg ml^{-1} soybean trypsin inhibitor, 0.5 mM PMSF, 50 mM NaF, 0.5 mM Sodium Ortho-Vanadate. The protein/antibody mixture was incubated with protein A Sepharose for 1 h and the immunoprecipitates were then washed twice with lysis buffer and four times with kinase buffer (50 mM Tris HCl pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg ml^{-1} BSA). Immunoprecipitates were then incubated with kinase buffer containing 5 μg histone H1, 60 μM cold ATP and 5 μCi of [^{32}P]γATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then analysed on a 13% SDS–PAGE gel. The gel was then stained, destained, dried and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised and radioactivity was measured by scintillation counting.

Reverse transcription-polymerase chain reaction amplification (RT-PCR)

RNA was isolated from cell lines and tissue samples as previously described (Keyomarsi and Pardee, 1993). To

remove chromosomal DNA contamination from RNA, 50 μg of total cellular RNA was incubated for 30 min at 37°C with 10 units of RNasin (Promega) and 20 units of RQI DNase (Promega) in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. After extraction with phenol/$CHCl_3$ (1:1) followed by $CHCl_3$, the supernatant was ethanol precipitated in the presence of 0.3 M NaOAC and RNA was redissolved in 0.1 × Tris-EDTA in diethyl pyrocarbonate-treated water. Reverse transcription was performed by incubating 1 μg of the DNase treated RNA with 300 units of Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT) (Gibco/BRL) in the presence of 15 μM oligo-dT (12–18) (Pharmacia) as a primer and 20 μM dNTP for 10 min at room temperature, 45 min at 42°C, 5 min at 99°C and 5 min at 5°C in the Gene Amp PCR system 9600 (Perkin Elmer Cetus, San Diego, CA). One half of the reaction was subsequently used for 30 cycles of PCR amplifications using GeneAmp PCR reagent kit (Perkin Elmer Cetus). PCR cycles include denaturation for 40 s at 94°C, annealing for 1 min at 61°C and polymerization for 1 min at 72°C. A minimum of three independent PCR amplifications from each specimen, for each experiment, were performed to guard against potential errors due to Taq polymerase misincorporation.

Oligonucleotides, cloning and sequencing of RT-PCR products

A pair of primers L1CYCE: 5′-GGGATGCGAAGGA-GCGGGACA-3′ and R1CYCE: 5′-AGCGGCGCAAC-TGTCTTTGGT-3′ based on the mRNA sequence of cyclin E (Koff *et al*., 1991; Lew *et al*., 1991) were designed to amplify the entire cyclin E coding sequence of human cyclin E cDNA (1250 bp, i.e. from nucleotide −23 to + 1227). To specifically amplify the cyclin E transcripts harboring the Δ9 and Δ148 deletion, the following sets of primers were used respectively: LMEMARK3: 5′-GC-AAACGTGACCGTTG-3′ and R1CYCE: 5′-AGCGGCG-CAACTGTCTTTGGT-3′; L1CYCE: 5′-GGGATGCGA-AGGAGCGGGACA-3′ and RMEMARK3: 5′-ACCG-CTCTGTGCTTCATC-3′. The PCR products were visualized by fractionating 1/5th of each reaction on a 1.5% agarose gel stained with ethidium bromide. A fraction of each reaction was then used to clone the RT-PCR products into the PCR II vector using the TA cloning system from Invitrogen (San Diego, CA). Plasmid DNA sequencing of cloned cDNA products with either T7 of SP6 primer was carried out using Sequenase 2.0 sequencing kit from United States Biochemicals Co (Cleveland, OH). Fifteen clones from each independent RT-PCR reaction (at least three) were completely sequenced in both orientations to confirm the sequences for Δ9 and Δ148 variants of cyclin E.

In vitro *translation*

To transcribe and translate the cyclin E cDNAs cloned in the PCR II vector we used the TNT coupled Reticulocyte Lysate system (Promega). Briefly, 1 μg of PCR II vector containing either cyclin E-wt, cyclin E-Δ9 or cyclin E-Δ148 was added to rabbit reticulocyte lysate (50% of total volume) in the presence of T7 RNA polymerase, 1 mM amino acid mixture minus methionine, 40 μCi [^{35}S]methionine (Dupont), RNasin ribonuclease inhibitor and the TNT reaction buffer (Promega) in a total volume of 50 μl. For non-radioactive reactions, unlabeled methionine was added to the mix and radioactivity was excluded. The reactions were then incubated at 30°C for 2 h and the translated radioactive products were separated by SDS–PAGE. The gels were then stained, destained, fluorographed, dried and the protein products were visualized by autoradiography. For visualization of the non-radioactive samples, the translation products were subjected to Western blot analysis using a polyclonal antibody specific to cyclin E.

Production of cyclins and kinases in insect cells

The cDNAs of cyclin E wild type, cyclin E-Δ9, cyclin E-Δ148 and cdk2 were subcloned into pMP3 (Pharmingen) plasmid containing the Basic Protein promoter that is active during and after viral DNA synthesis when the cell is producing baculovirus components to assemble the virus particles. At this stage there are larger numbers of modifying enzymes present which will increase the effectiveness of post-translational modification of the gene product of interest. Once the plasmids were constructed, they were individually co-transfected in sf9 insect cells with the linearized BaculoGold (Pharmingen) virus DNA (containing a lethal deletion), which through recombination would only produce viable recombinant baculovirus expressing our clones. The titer of all the supernatants were determined and insect cells were then infected with a plaque-forming unit/cell number of 1.

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Classification : Cell Biology

Cyclin E, a Redundant Cyclin in Breast Cancer

Julie Gray-Bablin[1], Juan Zalvide[2], M. Pat Fox[1], Chris J. Knickerbocker[1], James A, DeCaprio[2], and Khandan Keyomarsi[1]*

[1]Division of Molecular Medicine, Laboratory of Diagnostic Oncology, Wadsworth Center, Albany, NY 12201; [2]Division of Neoplastic Disease Mechanisms, Dana Farber Cancer Institute, Boston, MA 02146

*Corresponding author:

Khandan Keyomarsi, Ph.D.
Wadsworth Center
Empire State Plaza
P.O. Box 509
Albany, NY 12201
(518) 486-5799
FAX (518) 486-5798

email: keyomars@wadsworth.org

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Abstract:

Cyclin E is an important regulator of cell cycle progression that together with cyclin-dependent kinase 2 (cdk2) is crucial for the G1/S transition during the mammalian cell cycle. Previously, we showed that severe overexpression of cyclin E protein in tumor cells and tissues results in the appearance of lower molecular weight isoforms of cyclin E which together with cdk2 can form a kinase complex active throughout the cell cycle. In this study we report that one of the substrates of this constitutively active cyclin E/cdk2 complex is pRb in populations of breast cancer cells and tissues which also overexpress p16. In these tumor cells and tissues, we show the expression of p16 and pRb are not mutually exclusive. Overexpression of p16 in these cells results in sequestering of cdk4 and cdk6, rendering cyclin D1/cdk complexes inactive. However pRb appears to be phosphorylated through out the cell cycle following an initial lag revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes which functionally inactivate pRb. In addition the constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor but not the normal cell cycle. These observations suggest that overexpression of cyclin E in tumor cells which also overexpress p16, can bypass the cyclin D/cdk4-cdk6/p16/pRB feedback loop, providing yet another mechanism by which tumors can gain a growth advantage.

Introduction:

Progression through the eukaryotic cell cycle is mediated both positively and negatively by a variety of growth regulatory proteins (1-3). Cyclins and their catalytic cyclin dependent kinase (cdk) partners act positively to propel a cell through the proliferative cycle (4, 5). Activation of cyclin-cdk complexes results in a cascade of protein phosphorylations which ultimately induce cell cycle progression (1, 4). Although the identity of downstream substrates and effectors of cyclin-cdks remains to be firmly established, it is commonly believed cdk mediated phosphorylations manifest cell cycle regulation via inhibition of growth inhibitory signals and activation of proteins necessary for each stage of the cell cycle (6). A putative, well characterized substrate for the G1 cyclins is the retinoblastoma susceptibility gene product, pRb (7, 8). This protein is sequentially phosphorylated during the cell cycle presumably through the concerted activity of different cyclin-cdk complexes (9-11). This phosphorylation is required for cell cycle progression and the hypophosphorylated form of pRb inhibits cell cycle progression by tethering and inactivating transcription factors of the E2F family which are required for the trans-activation of S phase specific proteins including dihydrofolate reductase, cyclin A, and thymidylate synthase (12-14). The phosphorylation of pRb results in the release of E2F transcription factors freeing them to stimulate transcription of growth promoting target genes.

Inhibition of pRb phosphorylation, therefore, represents a potent form of growth inhibition. Such inhibition has recently been exemplified through the characterization of cyclin dependent kinase inhibitor proteins (CKIs) [Reviewed in: (15, 16)]. To date these proteins exist as two functionally and structurally distinct groups typified by p21 and its homologues p27 and p57 as well as p16 and p15 and their related homologues (17, 18). As potential tumor suppressors, the CKI genes have been extensively studied to evaluate the possible contribution of CKI specific genomic mutations to neoplastic transformation (17). In particular, the gene encoding p16, or MTS1 (multi tumor suppressor 1), on chromosome 9p21 has been postulated to encode a tumor suppressor and demonstrated to be mutated in a wide variety of tumor-derived cell lines (19-22).

A curious finding has ensued from the analysis of p16 in cancer; although both pRb and p16 are often mutated in human cancers, these mutations seem mutually exclusive (23-26). This inverse correlation has been established in various tumor cell types both *in vitro* and *in vivo*. A logical conclusion then is that these proteins, which act similarly to inhibit cell cycle progression, are differentially regulated by a common pathway, perhaps invólving a negative feedback loop. In fact, the growth suppression mediated via p16 overexpression has been shown to be definitively correlated with pRb status (27, 28). Thus p16 proliferative inhibition is only evident in cells expressing wild type pRb. As an inhibitor of the putative pRb kinases, cdk4 and cdk6, p16 is thought to bind, inhibit, and sequester these cdks thereby rendering cyclin D orphan with respect to cdk association. Some groups have postulated that p16 expression is regulated by pRb or by a feedback mechanism involving pRb (29) and it has been demonstrated by others that p16 is transcriptionally regulated by pRb (30). Such a mechanism would permit high levels of p16 to be expressed only when pRb is inactivated, by hyperphosphorylation, genomic mutation, or association with transforming viral oncoproteins. Although not without exception, the inverse correlation of these two proteins, particularly in breast epithelial cells, may represent a tightly regulated feedback mechanism.

In this report, we have identified and characterized an exception to the pRb/p16 inverse correlation rule. In the cell line MDA-MB-157 pRb is wild type and phosphorylated, p16 is significantly overexpressed and effectively binds cdk4 and cdk6 thus preventing cyclin D1 from binding to these kinases. We also have demonstrated that although cyclin D1-cdk4 and -cdk6 complexes are inactivated by p16, pRb is progressively synthesized and phosphorylated during the cell cycle. Cyclin D1, cdk4, and cdk6 are not overexpressed in this cell line, however, cyclin E is overexpressed and its levels and associated kinase activity remain constitutively high during all phases of the cell cycle. Additionally cyclin E-cdk2 complex can phosphorylate GST-Rb throughout the cell cycle. We conclude, therefore, that there exists a functional redundancy amongst the cyclins, such that overexpression of cyclin E may compensate for the inactivation of cyclin D complexes by p16 with respect to the pRb phosphorylation and cell cycle progression.

Results:

Overexpression of p16 and absence of cyclin D1/Cdk4 -cdk6 complexes, in a breast cancer cell line with functional retinoblastoma protein: A panel of 13 breast cell lines were surveyed for the correlation of p16 and Rb status as well as association of p16 and cyclin D1 with cdks 4 and 6 (Figure 1). The cell lines used include three proliferating normal mammary epithelial cell strains obtained from reduction mammoplasties and used at early passages, one near diploid normal-immortalized breast epithelial cell line and 9 tumor cell lines with different estrogen receptor, and p53 status, and cyclin E levels as outlined in table 1.

We examined the expression of pRb by direct immunoblotting with a monoclonal antibody where the presence of functional pRb is inferred from the presence of higher molecular weight hyperphosphorylated forms of the protein. These analysis revealed that besides three tumor cell lines (figure 1A, lanes 8, 11 and 12-i.e MDA-MB-436, HBL-100, and Hs-578T), where pRb is either mutated (31), inactive due to its binding to SV40 large T-antigen, or not expressed, pRb is present and functional in all the other cell lines examined. Furthermore, in all the pRB positive cell lines, there are at least two pRb bands present representing different phosphorylation states of pRb. (Due to different levels of pRb expression in each of the cell lines longer exposures were used to evaluate presence of slower migrating/functional form of pRb-specifically in lanes 1, 2 and 5- data not shown). Next, we correlated the expression of p16 levels with pRb status and found that p16 is overexpressed in three cell lines (figure 1A, lanes 6, 8 and 11), two of which Rb has been functionally compromised (i.e. MDA-MB-436 and HBL-100). Curiously, in MDA-MB-157 which contains a wild-type pRB, p16 is also markedly overexpressed (figure 1A, lane 6). Hence MDA-MB-157, in which cyclin E is severely overexpressed [Table 1, (43)], is one exception to the reciprocal p16/Rb correlation rule.

Since, overexpression of cdk4, cdk6 or cyclin D1 could counteract the inhibitory effect caused by the over-abundance of p16, we also measured the relative levels of these proteins in all 13 cell lines (Fig. 1A). Western blot analysis with cyclin D1, cdk4, and cdk6 revealed that these proteins were not overexpressed in MDA-MB-157 cell line relative to the other 12 cell lines

examined, suggesting that the overexpressed p16 may adequately sequester cdk4 and cdk6 away from cyclin D1, rendering it inactive. To test this hypothesis we performed a series of 2 step immunoprecipitations followed by Western blot analysis (Fig. 1B). When p16 immunoprecipitates were separated on denaturing gels, transferred to PVDF membrane, and blotted with antiserum to cdk4 or cdk6, p16 was capable of forming a complex with both cdk4 and cdk6 in the three tumor cell lines where p16 is overexpressed. Curiously, p16 was also capable of forming a complex with cdk6 in normal breast cell strains where no overexpression of p16 or cdk6 were noted. However, cyclin D1 immunoprecipitates which were separated and blotted with antibodies to cdk4 or cdk6 revealed that in the normal cell strains cyclin D1 formed a complex with cdk4 and cdk6 suggesting that p16 did not completely sequester these kinases from cyclin D1. On the other hand, in tumor cells where p16 is overexpressed, no complexes were formed between cyclin D1 and cdk4 or cdk6, suggesting that in these three tumor cell lines enough p16 is overexpressed to sufficiently sequester cdk4 and cdk6 away from cyclin D1 preventing it from forming complexes with these kinases (Fig. 1B). Collectively these data provide evidence for the absence of cyclin D1/CDK complexes in a breast cancer cell line with a functional retinoblastoma protein.

Cyclin E associated kinase phosphorylates pRb in the absence of cyclin D1/cdk4 or cyclin D1/cdk6 complexes in tumor cells: To examine the cell cycle regulation of pRb in normal and tumor cells we synchronized both cell lines by double thymidine block and analyzed the pattern of pRb expression and phosphorylation by Western blot analysis (Fig 2A). Synchrony of both cell types at several times after release from the block was monitored by flow cytometry (Fig 2C). At various times after release from treatment for synchronization, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to pRb, cyclins E and A (Fig 2A). In normal 76N cells, the pattern of synthesis and phosphorylation of pRb as well as expression of cyclin E and cyclin A proteins is consistent with that seen for other normal cell types with levels rising prior to S phase and oscillating thereafter in the cell cycle (8, 32, 33). In addition pRb is present mainly in the hyperphosphorylated form at G1/S boundary up to G2, where the

levels drop, to resume again at G1. Furthermore, there is only one major form (i.e., 50KDa) of cyclin E protein detected. However, in the tumor cells, pRb and cyclin E proteins do not appear to be cell cycle regulated. pRb is induced and phosphorylated shortly after release from thymidine block and remains in that phosphorylated state through out the cell cycle. In addition, multiple isoforms of cyclin E protein are present with similar signal intensities and banding patterns during the time intervals examined. In the same tumor cell extracts, cyclin A protein is cell cycle regulated with peak levels coinciding with peak S and early M phase. Hence, it appears that in this tumor cell line, pRb and cyclin E are abnormally regulated during the cell cycle.

To decipher whether cyclin E-associated kinase is responsible for the phosphorylation of pRb, cells were immunoprecipitated with cyclin E antibody and used in kinase assays with either histone H1 or a recombinant GST-Rb fusion protein as substrates. In normal cells, cyclin E associated kinase is capable of phosphorylating histone H1 and is cell cycle regulated, coinciding with the levels of cyclin E protein expression (Fig 2A). However the same cyclin E immunoprecipitates prepared from normal cells were not capable of phosphorylating GST-Rb. In tumor cells, on the other hand, cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle resulting in a constitutive pattern of histone H1 and GST-Rb phosphorylation. Lastly, the timing of pRB expression in the tumor cell cycle (Fig. 2A) is similar to the timing of phosphorylation of GST-Rb by cyclin E immunoprecipitates. These observations suggest that overexpression of cyclin E results in an active kinase complex throughout the cell cycle capable of not only phosphorylating histone H1, but also GST-Rb. Hence in tumor cells which overexpress p16, resulting in the inactivation of cyclin D1/CDK4 or cyclin D1/cdk6 complexes, pRb can still get phosphorylated by cyclin E/associated kinase.

Overexpression of cyclin E and p16 in breast tumor tissues is correlated with functional pRb: Since the lack of inverse association of pRb and p16 was observed in only one of 3 breast tumor cell lines overexpressing p16 (Figure 1A), we were interested in deciphering the frequency at which such a phenomena would occur in breast tissue samples. As such we

examined 20 tumor tissue specimen obtained from breast cancer patients. Table 2 lists estrogen and progesterone status, ploidy, and proliferation index expression as measured by immunoflouresence with the respective antibodies followed by image analysis as previously described (34, 35). We also analyzed the expression of cyclin E, p16 and pRb in these samples by Western blot analysis. The results revealed that cyclin E was severely overexpressed and present in lower molecular weight forms in 18/20 tissue samples which is consistent with the role of cyclin E as a prognosticator for breast cancer (36-38). The pattern of cyclin E expression observed in these tumor specimen were similar to those used in a previous study (50) showing presence of lower molecular weight forms of cyclin E with increasing stage of the disease. Interestingly most of the tumor specimen which showed an overexpression of cyclin E also were negative for ER and PR. A negative steroid receptor status is indicative of poor response to endocrine and cytotoxic chemotherapy characteristics of very aggressive breast tumors (39). Furthermore p16 was overexpressed in 7 (i.e KK-005, 086, 147, 173, 190, 369, and 399) out of the 20 samples examined. Three of these 7 samples had a defect in pRb expression, while in the remaining 4 samples (i.e KK-005, 147, 173, and 369) pRb was expressed and present in multiple bands, suggesting a functional protein. In addition cyclin E was severely overexpressed in all 4 p16/pRb double positive samples. Hence, these observations suggest that *in vivo*, in breast cancer tissues which overexpress cyclin E, overexpression of p16 is not always accompanied by a defect in pRb, consistent with results obtained with MDA-MB-157 cell line. Cyclin E which is overexpressed and present in lower molecular weight forms in these tumor tissue samples may be capable of phosphorylating pRb in the absence of functional cyclin D containing complexes *in vivo* as well as in cell lines.

Cyclin E is present in E2F complexes throughout the cell cycle of tumor but not normal cells. One of the major targets of growth regulation by pRB is the E2F family of transcription factors. During the G1 phase of the cell cycle, underphosphorylated pRB binds to E2F and represses its transcriptional activity. Phosphorylation of pRB by cyclins during late G1 and S phase release E2F, that in turn leads to activation of the transcription of genes important for

cell cycle progression. Similarly, p107 and p130, two pRB-related proteins, regulate the transcriptional activity of E2F. In addition, both cyclin A and E can bind to p107 and p130 while in complex with E2F. While the significance of this association is not known, it has been suggested that it regulates the transcriptional activity of E2F.

To determine, whether the cyclin E overexpression in the tumor cell lines affected the E2F DNA binding complexes throughout the cell cycle, we performed bandshift assays using an oligonucleotide with an E2F binding site as a probe. As a control, extracts from a synchronized population of normal cells were prepared. As described previously (13), normal cells contained several E2F complexes that were present at various times in the cell cycle. The disappearance of E2F complexes at 6, 9 and 12 hours after release from the thymidine block occurred when the cells were enriched for G2/M (13) (figure 3A). The complex marked with an arrow contained the pRB-related protein p107 and cyclin A, as shown by antibody supershift analysis (data not shown). Addition of cyclin E antibody did not have any effect on the mobility of this complex (figure 3A), suggesting that cyclin E is not the predominant cyclin in the p107/E2F complex in normal cells. On the other hand, in extracts prepared from tumor cells, E2F complexes were present throughout the cell cycle and no loss of these complexes was observed during G2/M. The complex marked with an arrow could be disturbed with anti-p107 and partially with anti-cyclin A antibodies (data not shown). The addition of an anti-cyclin E antibody resulted in a super shift of a large proportion of the complex, suggesting that most of the p107-E2F complex contained cyclin E (figure 3B). Addition of antibodies to cyclin A and cyclin E to the same extract did not result in the appearance of any different complexes than when both antibodies were added independently (data not shown), suggesting that both cyclins did not form part of the same complex. The association of cyclin E with the E2F complexes in tumor cells paralleled the constitutive expression of cyclin E throughout the cell cycle (Fig 2A, right panel). Hence, overexpression of cyclin E in tumor cells was capable of forming a major complex with p107 and E2F. This is a second example of how overexpression and constitutive expression of cyclin E could result in a dual role for this cyclin allowing redundancy in function.

Methods

Cells lines, culture conditions, and tissue samples. The culture conditions for all cell lines used in this study were described previously (38, 40). Snap frozen surgical specimens from patients diagnosed with breast cancer were obtained from the Quantitative Diagnostic Laboratories (Almhurst, Illinois). 76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were synchronized at the G1/S boundary by a modification of the double thymidine block procedure (41) as described previously (40). For each time interval 10^6 cells were subjected to FACScan analysis as previously described (40, 42).

Western blot and Immune complex kinase analysis Cell lysates and tissue homogenates were prepared and subjected to Western blot analysis as previously described (37, 38). Primary antibodies used were monoclonal antibodies to cyclin E and D1 (Santa Cruz Biochemicals, Santa Cruz, CA), cdk4 (Transduction Laboratories, Lexington, KY), pRb (PharMingen, San Diego, CA), and p16 (Jim DeCaprio); polyclonal antibodies to cdk6 (Santa Cruz Biochemicals, Santa Cruz, CA) and cyclin A (a gift form J.W. Harper, Baylor College of Medicine). Immunoprecipitations and H1 kinase assays were performed as previously described (40, 43). Briefly, for H1 kinase and GST-Rb kinase assays 500 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with polyclonal antibody to cyclin E. Immunoprecipitates were then incubated with kinase buffer containing either 5ug histone H1 or 1 ug purified GST-RB, 60 μM cold ATP, and 5μCi of [^{32}P] γATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then run on a 13% SDS-PAGE gel. The gel was then stained, destained, dried and exposed to X-ray film.

For Immunoprecipitation followed by Western blot analysis, 250 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with either monoclonal antibody to p16, polyclonal antibody to cyclin D1 obtained from Dr. M. Pagano (Mitotix, Cambridge, MA; (44)), or monoclonal antibody to cyclin D1-clone HD33 (a gift from E. Harlow and C. Ngwu, Massachusetts General Hospital [MGH] Cancer Center) in lysis buffer as

described above. The Immunoprecipitates were then electrophoresed on a 13% SDS-PAGE, transferred to Immobolin P, blocked and incubated with either polyclonal antibody to cdk4 obtained from Dr. M. Pagano (Mitotix, Cambridge, MA; (44)), or cdk6 as described in the figure legend.

Gel Retardation assays: Whole cell extracts were prepared as previously described (37, 38) and 15 μg of protein were used per lane. Binding reactions were performed as described elsewhere (13, 45). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human DHFR promoter (DHFR WT) (13). For antibody perturbation experiments, 2 μl (200ng) of rabbit polyclonal antibody to cyclin E (UBI) was added.

Discussion:

The interplay between cyclin D1/cdk4-cdk6/p16/pRB has been implicated as a crucial G1-phase controlling pathway which becomes frequently de-regulated in many types of cancer. Any mutations giving rise to an imbalance in any one of these proteins may therefore result in a cell growth advantage leading to tumorigenesis. In this model, overexpression of p16 would prevent cdk4/cdk6 from phosphorylating pRb, and lead to a G1 block (27-29). Thus, p16 is thought to negatively regulate the cell cycle (46). In fact, several studies have documented that primary tumors which showed expression of functional pRb protein did not express p16 protein (due to mutations in the gene) and conversely, cells that expressed p16 protein, did not have a detectable pRb protein (23-26). These studies suggest a link between D type cyclins, cdk4/cdk6, pRb and p16, such that overexpression of cyclin D1, inactivation of pRb or loss of p16 may have equivalent consequences for loss of normal growth control. In addition, this model predicts a lack of functional redundancy of this pathway with other cell cycle regulatory proteins.

Even though many studies have corroborated the p16/pRb inverse correlation model, there have also been documentation to the contrary. For example in their analysis of pRb and p16 expression in lung cancers, Otterson et al.(25) reported that 14% of Small Cell Lung Cancers and

15% of Non-Small Cell Lung Cancers (NSCLC) examined were p16 and pRb double positives, and Sakaguchi et al. (47) reported that 16.4 % of NSCLC studied immunohistochemically also stained positively for both p16 and Rb protein. In addition Gerardts et al. (48) report that in 43% of all carcinomas examined (i.e. breast 5/20, bladder 7/19, colon 16/19, and lung 4/17), both pRb and p16 could be detected suggesting that in common human malignancies p16 and pRb expression is not mutually exclusive. Furthermore, Musgrove et al. (49) report that in 50% of breast cancer cell lines examined INK4p16 mRNA was expressed in the absence of any pRb mutations. Lastly Ueki et al. (50) show that 13% of glioblastoma cell lines examined showed neither p16 nor RB alterations and Wang et al. (51) report that regardless of the status of p16 protein, all 15 melanoma cell lines examined showed the presence of pRb protein ruling out an inverse correlation between the expression of p16 and pRb in these particular cell lines.

One possible explanation for the lack of inverse correlation between p16 and pRb may be due to overexpression of cyclin E which could act redundantly and replace cyclin D/cdk complexes for phosphorylating pRb. In concordance with this redundancy hypothesis Hinds et al. (52) first demonstrated that overexpression of several different cyclins, including cyclin E, could override the growth arrest properties of pRB in SaOS-2 cells. In addition we had previously reported that cyclin E is severely overexpressed in all breast cancer cell lines examined (38) and overexpression of cyclin E is accompanied by its constitutive expression and activity throughout the tumor cell cycle (40). Since cyclin E is overexpressed and forms a complex with cdk2 constitutively, the active complex can act upstream of pRb and phosphorylate it even when cyclin D is inactive due to overexpression of p16. To test this model, in this study we used a breast cancer cell line which exemplified an exception to the inverse correlation rule of p16/pRb. In this tumor cell line (i.e. MDA-MB-157) cyclin E is markedly overexpressed and present in lower molecular weight isoforms, p16 is also overexpressed and pRb is not mutated and detectable in both its hypo- and hyperphosphorylated forms. Under these conditions we show that p16 binds to both cdk4 and cdk6 and inhibits the binding of cyclin D1 to these cdks. We also provide evidence that in synchronized populations of MDA-MB-157 cells pRb is phosphorylated through out the cell cycle

following an initial lag revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. These analysis suggest that cyclin E/cdk2 and not cyclin D/cdk4-cdk6 is a candidate kinase complex capable of phosphorylating pRb through out the cell cycle of this tumor cell line.

To directly examine the lack of inverse correlation of p16 and pRb in vivo we document in Table 2 that in breast tumor specimen obtained from breast cancer patients where cyclin E is markedly overexpressed, and p16 is also overexpressed, pRb is detectable in both its hypo and hyperphosphorylated forms. These studies suggest that phosphorylation of pRb under conditions where cyclin D/cdk complexes are rendered inactive is not an artifact of the culture conditions and occurs in vivo.

Since cyclin E is constitutively expressed in MDA-MB-157 cancer cells, and is present during times in the cell cycle when cyclin A is not detected (see figure 2), it followed that cyclin E could also replace cyclin A containing complexes. In fact as displayed in Figure 3 cyclin E can function redundantly and replace cyclin A in E2F complexes with cdk2 and p107 in tumor cells. In normal cells, cyclin E was found in complex with the pRB-related proteins p107 and p130 and E2F during the late G1 and early S phase of the cell cycle. We have found that while this cyclin was a minor component of E2F DNA binding complexes in normal cells, it was a major component of this complex in MDA-MB-157 cells. Interestingly, while normal cells display a down regulation of E2F DNA binding activity in the G2/M phases of the cell cycle, MDA-MB-157 cells show constitutive E2F DNA binding complexes through the cell cycle. This raises the possibility that overexpression of cyclin E perturbed the regulation of E2F activity not only by promoting the hyperphosphorylation of pRB but also by perturbing the cell cycle regulation of E2F by p107.

Based on our observations in breast cancer cell lines and tumor tissue samples we suggest an alternative order of events along the G1 phase controlling pathway culminating in phosphorylation of pRB. In this pathway cyclin E would act upstream of pRb bypassing cyclin D/cdk4 and giving the tumor cells a selective growth advantage even in the presence of high levels

of p16. Hence abrogation of cyclin D1, cdk4/cdk6, or p16 will not have any affect on the phosphorylation of pRb which will be accomplished by cyclin E/cdk2 in these cells leading to a deregulated progression through G1. Our data also demonstrates that cyclin D1 is not required for G1 progression in tumor cells which exhibit an overexpressed cyclin E and a wild-type pRB. As a result the function of cyclin D1 is dispensable not only in cell lines in which pRb is inactivated as described (53), but also in cell lines were cyclin E is overexpressed and constituitively active (this study and (54). Lastly, this study provides evidence for a lack of functional link between p16 and pRb suggesting that in sub populations of breast cancers pRB is not a major substrate for the inhibitory activity of the p16 product. Hence certain populations of tumor cells can overcome the role of p16 as a tumor suppressor protein by providing a redundant pathway to inactivate pRB and provide a growth advantage to the cells.

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Figure Legends:

Figure 1: Expression and complex formation of p16/pRB pathway proteins in normal and tumor derived breast epithelial cells. **(A)** Western blot analysis: Exponentially growing normal and tumor cells were subjected to Western blot analysis using 50µg protein for each cell line in each lane of either a 6% (pRb) 13% (cyclin D1, cdk4, and cdk6), or 15% (p16) acrylamide gel and blotted as described. The same blot was reacted with cyclin D1, cdk4 and cdk6 affinity purified antibodies. The blots were stripped between the three antibodies in 100 mM ß-mercaptoethanol, 62.5 mM Tris HCL (pH 6.8) and 2% SDS for 30 min at 55 °C. **(B)** Immune-complex formation: For immunoprecipitation followed by Western blot analysis equal amounts of protein (500 µg) from cell lysates prepared form each cell line were immunoprecipitated with either monoclonal antibody to p16 (p16/cdk4 and p16/cdk6), polyclonal antibody to cyclin D1 (cyclin D1/cdk4) or a monoclonal antibody to cyclin D1 (cyclin D1/cdk6), coupled to protein A/G beads and the immunoprecipitates were washed, boiled for 3 min, separated by SDS-13% PAGE, blotted to Immobilon membranes, and hybridized with either polyclonal antibody to cdk4 (p16/cdk4), polyclonal antibody to cdk6 (p16/cdk6 and cyclin D1/cdk6-arrow pointing to the complexed protein), or monoclonal antibody to cdk4 (cyclin D1/cdk4). The list of normal and tumor cell lines is presented in Table 1 using identical numbers.

Figure 2: Phosphorylation of pRb in synchronized population of tumor versus normal cells. Both cell types were synchronized by double thymidine block procedure. At the indicated times following release from double thymidine block, cell lysates were prepared and subjected to **(A)** Western blot and **(B)** Histone H1 or GST-Rb kinase analysis. Protein (50µg) for each time point was applied to each lane of either a 6% (pRB) or 10% (cyclins E and A) acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity purified polyclonal antibodies. The blots were stripped between the two assays as described for figure 1. For kinase activity, equal amount of proteins (600 ug) from

cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) coupled to protein A beads using either histone H1 or purified GST-Rb as substrates. **(C)**: The relative percentage of cells in different phases of the cell cycle for each cell line at various times after release from double thymidine block was calculated from flow cytometric measurements of DNA content. (◆) cells in S phase, (o) cells in G2/M phase, and (❑) cells in G1 phase.

Figure 3: Cyclin E is the predominant cyclin in p107/E2F complexes in tumor cells: E2F complex were analyzed by gel retardation assays using cell lysates (15μg) prepared from synchronized populations (see figure 2) of **(A)** normal 76N and **(B)** of tumor MDA-MB-157 cells. The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human DHFR promoter. 200ng of the anti-cyclin E antibody was used to disrupt the E2F complexes.

Table 1: Characterization of normal and tumor-derived breast epithelial cells

Cell Lines	Cell Types	Estrogen Receptor (38)	p53	Cyclin E (38,40)	pRB*
1 - 70N	N-mortal	-	+ (55)	+	+
2 - 81N	N-mortal	-	+ (55)	+	+
3 - 76N	N-mortal	-	+ (55)	+	+
4 - MCF-10-A	N-immortalized	-	+ (56)	+	+
5 - MCF-7	A (pe)	+	+ (56)	+++	+
6 - MDA-MB-157	C (pe)	-	- (56)	++++++	+
7 - MDA-MB-231	A (pe)	-	- (56)	++++	+
8 - MDA-MB-436	A	-	- (57)	+++++	-
9 - T47D	DC (pe)	+	- (56)	++	+
10 -BT20	C	+	+ (56)	++	+
11 - HBL-100	T (bm)SV40 transformed	-	- (56)	+++	-
12 - HS-578T	DC	-	- (56)	++++	-
13 - ZR75T	IDC	+	+ (56)	+++	+

N, normal breast cells from reduction mammoplasty; A, adenocarcinoma; pe, pleural effusion; C, carcinoma; DC, ductal carcinoma; T(bm), tumor breast milk); IDC, infiltrating DC. Cell type, Estrogen receptor (ER), p53 and Cyclin E status as determined in indicated references. + indicates wild type, ++(++++) indicates various degrees of overexpression with MDA-MB-157 which showing the highest degree (64 fold, hence 6 +s) of cyclin E overexpression. - mutant or not expressed. * pRB status is adopted from figure 1, where + indicates wild-type and present in hypo- and hyper-phosphorylated forms and - indicates mutated or virally bound and inactive.

Table 2: Correlation of p16 and pRb status in a series of breast carcinomas

Patient ID #	ER/PR[1]	DNA Index/ Ploidy[1]	Proliferation Index (%)[1]	Cyclin E[2]	p16[2]	pRb[2]
KK005	-/-	1.18/Aneuploid	12.2 (H)	+++	++++	+
KK017	-/-	1.72/Aneuploid	1.5 (L)	++++++	±	+
KK020	-/-	1.73/Aneuploid	14.1 (H)	+++++	-	-
KK036	+/-	1.84/Tetraploid	3.3 (L)	++	±	+
KK061	-/-	ND	ND	++++	±	-
KK070	+/+	ND	ND	+	±	-
KK076	-/-	2.08/Tetraploid	12.5 (H)	+++	±	-
KK086	-/-	1.50/Aneuploid	36.0 (H)	+++++	++	-
KK147	ND	ND	ND	+++++	+++	+
KK173	+/-	1.91/Tetraploid	30.2 (H)	+++++++	++++	+
KK190	-/-	2.09/Tetraploid	31.8 (H)	+++++++	+++	-
KK322	+/-	2.70/Aneuploid	30.0 (H)	+++	-	+
KK369	ND	ND	40.0 (H)	+++++++	++++	+
KK399	-/-	ND	ND	++++	++++	-
KK400	+/-	ND	ND	++++	±	-
KK407	-/-	1.89/Tetraploid	18.0 (H)	++++	-	-
KK428	-/-	1.75/Aneuploid	27.0 (H)	++++	-	-
KK429	-/-	1.71/Aneuploid	28.0 (H)	+++++	-	-
KK457	ND	ND	ND	++++++	-	+
KK458	-/-	1.96/Tetraploid	11.3 (H)	+	-	+

1: Quantitation of immunohistochemical staining by image analysis was performed on sections stained with either the monoclonal antibody to Estrogen Receptor, H222 (ER-ICA Kit, Abbott Laboratories, North Chicago, IL), monoclonal antibody to Progesterone receptor, mPRI, (Cell Analysis Systems, Inc,. Lombard, IL) or monoclonal antibody to Ki67 (DAKO-PC, Dako Corporation, Santa Barbara CA) as described (34, 35). KI67 staining determined growth fraction of the tumor. Values indicate percent positive staining: 1.0-7.0% is indicative of low (L) proliferation index, 7.1-11.9 is indicative of moderate (M) proliferation index and >12.0% is indicative of high (H) proliferation index. For each case the DNA ploidy was determined by quantitation of the DNA Feulgen stain by computerized microdensitometry as described (58).

2: Cyclin E, p16, and pRb levels were measured using Western blot analysis with HE12 monoclonal antibody to cyclin E (Santa Cruz Biotechnology, San Diego, CA) as described (38, 40), monoclonal antibodies to p16 and pRb as described in text. Levels of cyclin E in tumor tissue samples were correlated with 76N normal (+) and MDA-MB-157 (++++++) tumor cell lines. For example, cyclin E in MDA-MB-157 cell line is 64 fold (i.e. ++++++) overexpressed compared to 76N cell line (i.e. +) (38). Any tumor tissue overexpressing cyclin E more than MDA-MB-157 received 7 +s (i.e +++++++). P16 levels were also correlated with MDA-MB-157 (++++) cell line. Equal protein loading was monitored by reprobing blots with actin and all blots were analyzed by densitometry using AGFA scanner and IP Lab Gel software.

Figure 1

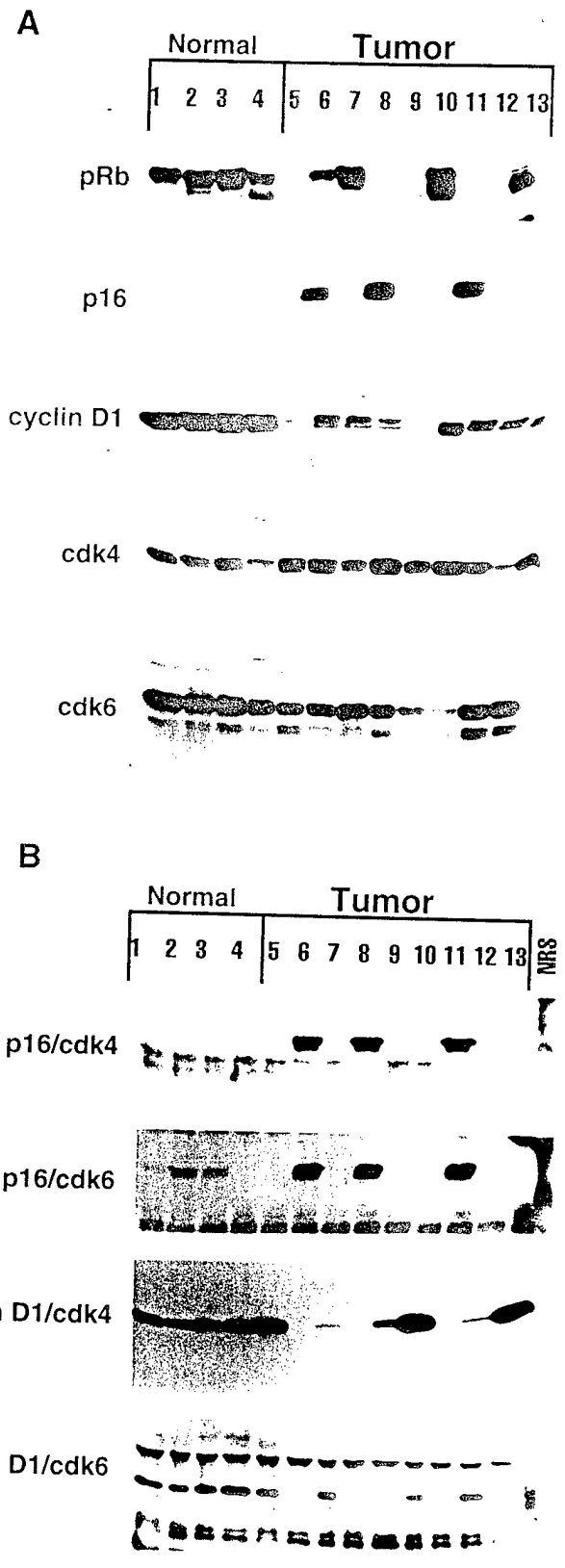

A
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13
pRb
p16
cyclin D1
cdk4
cdk6
B
Normal
Tumor
1 2 3 4 5 6 7 8 9 10 11 12 13 MRS
p16/cdk4
p16/cdk6
cyclin D1/cdk4
cyclin D1/cdk6

Figure 2

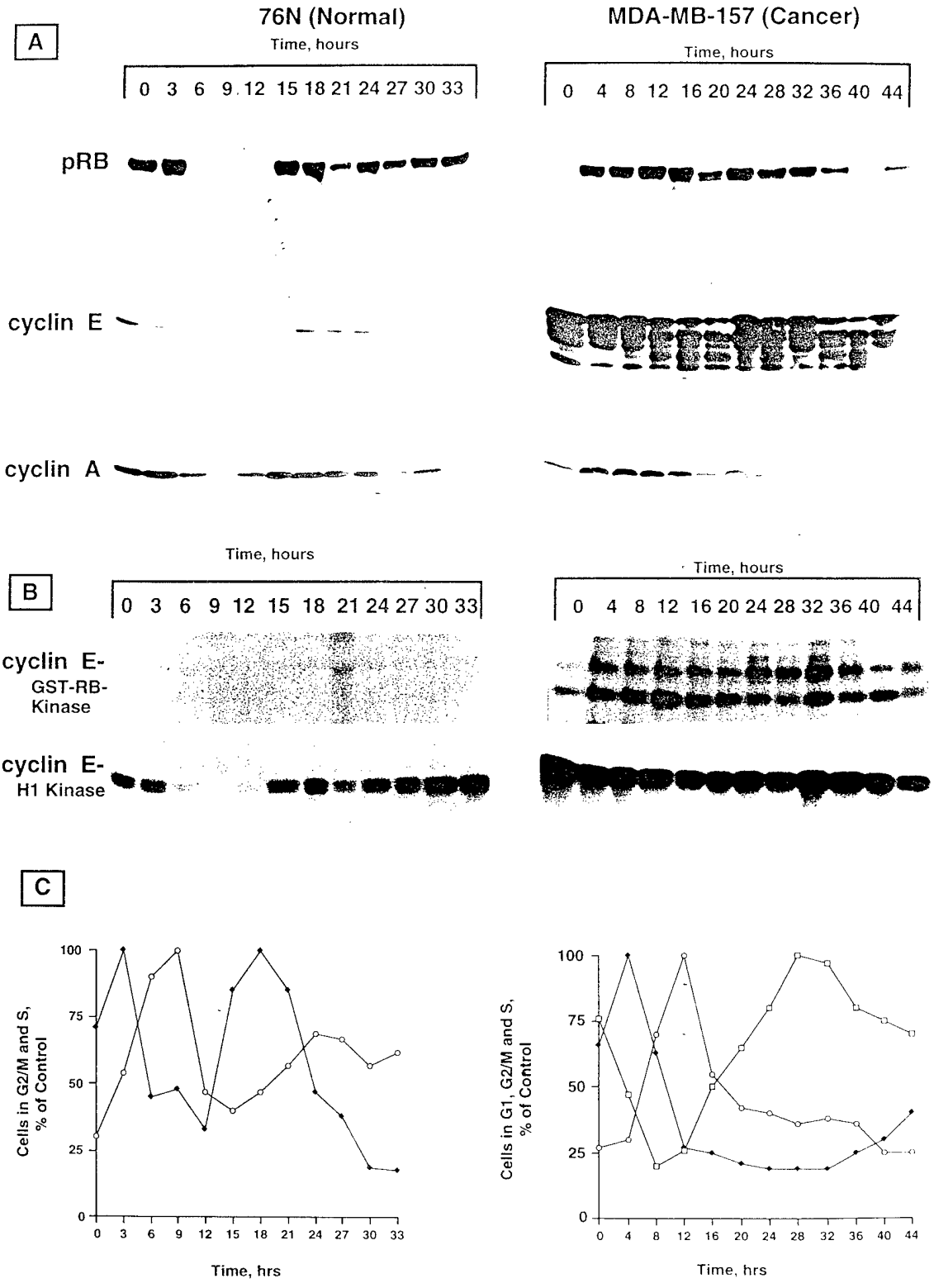
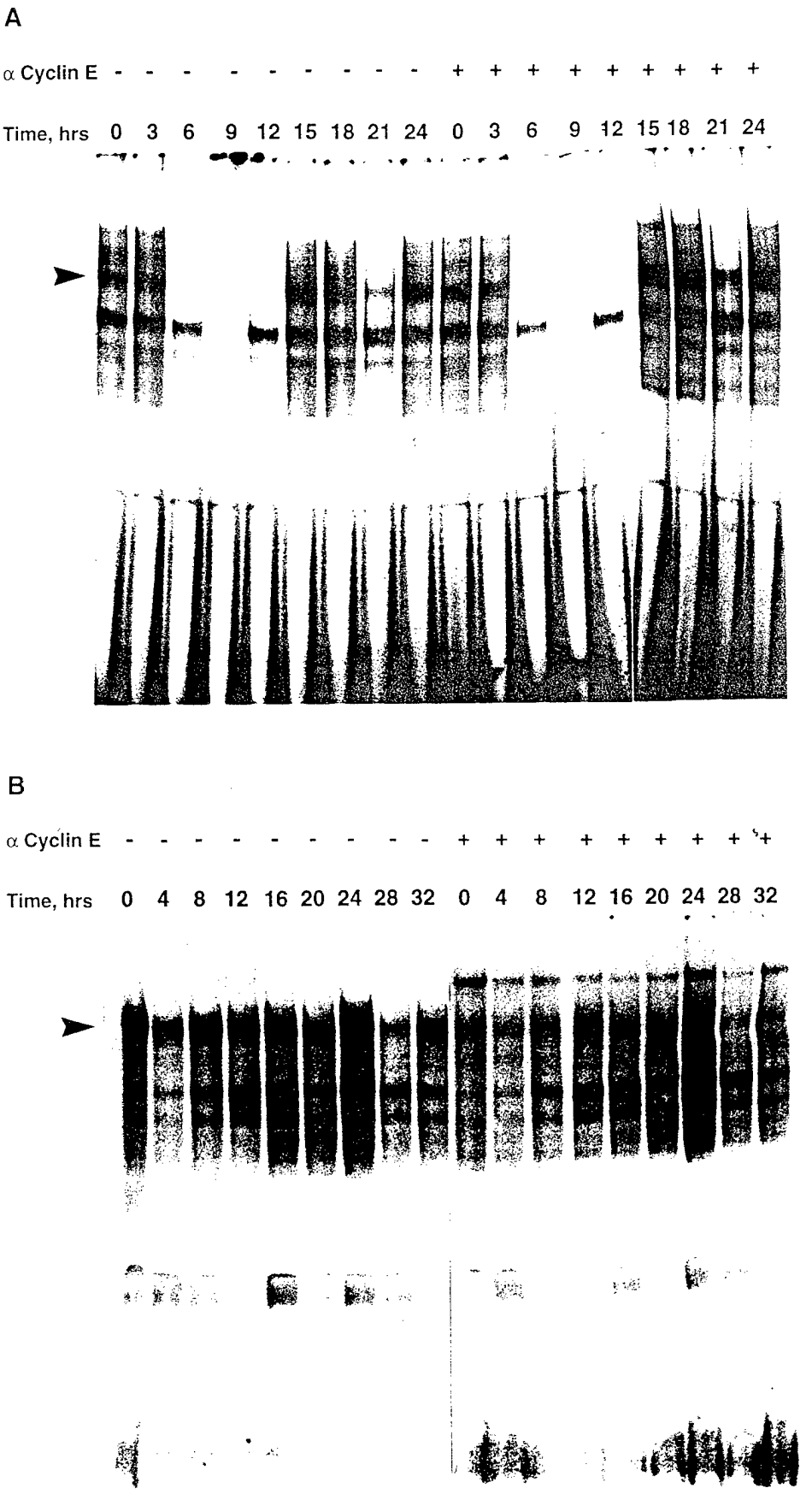

A
76N (Normal)
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
MDA-MB-157 (Cancer)
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
pRB
cyclin E
cyclin A
B
Time, hours
0 3 6 9 12 15 18 21 24 27 30 33
Time, hours
0 4 8 12 16 20 24 28 32 36 40 44
cyclin E-
GST-RB-
Kinase
cyclin E-
H1 Kinase
C
Cells in G2/M and S,
% of Control
100
75
50
25
0
0 3 6 9 12 15 18 21 24 27 30 33
Time, hrs
Cells in G1, G2/M and S,
% of Control
100
75
50
25
0
0 4 8 12 16 20 24 28 32 36 40 44
Time, hrs

Figure 3


A
α Cyclin E - - - - - - - - - + + + + + + + + +
Time, hrs 0 3 6 9 12 15 18 21 24 0 3 6 9 12 15 18 21 24
B
α Cyclin E - - - - - - - - - + + + + + + + + +
Time, hrs 0 4 8 12 16 20 24 28 32 0 4 8 12 16 20 24 28 32